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(71) Applicant *(for all designated States except US)*: **E. I. DU PONT DE NEMOURS AND COMPANY** [US/US]; 1007 Market Street, Wilmington, Delaware 19898 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **AUKERMAN, Milo J.** [US/US]; 1203 Delpa Drive, Newark, Delaware 19711 (US).

(74) Agent: **DRESS, Virginia M.**; Pioneer Hi-Bred International, Inc., 7100 N.W. 62nd Avenue, Johnston, Iowa 50131-1000 (US).

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(54) Title: GENE SILENCING

(57) **Abstract:** The invention provides methods and compositions useful in target sequence suppression and target sequence validation. The invention provides polynucleotide constructs useful for gene silencing, as well as cells, plants and seeds comprising the polynucleotides. The invention also provides a method for using microRNA to silence a target sequence.

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GENE SILENCING

Field of the Invention

The field of the present invention relates generally to plant molecular biology. More specifically it relates to constructs and methods to suppress the expression of targeted genes.

Background

Reduction of the activity of specific genes (also known as gene silencing, or gene suppression) is desirable for several aspects of genetic engineering in plants. There is still a need for methods and constructs that induce gene suppression against a wide selection of target genes, and that result in effective silencing of the target gene at high efficiency.

Brief Description of the Figures

Figure 1. Predicted hairpin structure formed by the sequence surrounding miR172a-2. The mature microRNA is indicated by a grey box.

Figure 2. miR172a-2 overexpression phenotype. a, Wild type (Columbia ecotype) plant, 3.5 weeks old. b, EAT-D plant, 3.5 weeks old. c, Wild type flower. d, EAT-D flower. Note absence of second whorl organs (petals). Arrow indicates sepal with ovules along the margins and stigmatic papillae at the tip. e, Cauline leaf margin from a 35S-EAT plant. Arrows indicate bundles of stigmatic papillae projecting from the margin. f, Solitary gynoecium (arrow) emerging from the axil of a cauline leaf of a 35S-EAT plant.

Figure 3. The EAT gene contains a miRNA that is complementary to APETALA2 (AP2). a, Location of the EAT gene on chromosome 5. The T-DNA insertion and orientation of the 35S enhancers is indicated. The 21-nt sequence corresponding to miR172a-2 is shown below the EAT gene (SEQ ID NO: 86). b, Putative 21-nt miR172a-2/AP2 RNA duplex is shown below the gene structure of AP2. The GU wobble in the duplex is underlined. c, Alignment of AP2 21-nt region (black bar) and surrounding sequence with three other Arabidopsis AP2 family members, and with two maize AP2 genes (IDS1 and GL15). d, Alignment

of miR172a-2 miRNA (black bar) and surrounding sequence with miR172-like sequences from *Arabidopsis*, tomato, soybean, potato and rice.

Figure 4. miR172a-2 miRNA expression. a, Northern blot of total RNA from wild type (lanes 3 and 7) and EAT-D (lanes 4 and 8). Blots were probed with sense (lanes 1-4) or antisense (lanes 5-8) oligo to miR172a-2 miRNA. 100 pg of sense oligo (lanes 2 and 6) and antisense oligo (lanes 1 and 5) were loaded as hybridization controls. Nucleotide size markers are indicated on the left. b, S1 nuclease mapping of miR172a-2 miRNA. A 5'-end-labeled probe undigested (lane 1) or digested after hybridization to total RNA from wild-type (lane 2), EAT-D (lane 3), or tRNA (lane 4).

Figure 5. Developmental expression pattern of miR172 family members. a, RT-PCR of total RNA from wild type seedlings harvested at 2, 5, 12, and 21 days after germination (lanes 1-4, respectively), or from mature leaves (lane 5) and floral buds (lane 6). Primers for PCR are indicated on the left. b, Northern analysis of mirR172 expression in the indicated mutants, relative to wild type (Col). Blot was probed with an oligo to miR172a-2; however, all miR172 members should cross hybridize.

Figure 6. Expression analysis of putative EAT target genes. a, Northern blot analysis of polyA+ RNA isolated from wild type (Col) or EAT-D floral buds. Probes for hybridization are indicated on the right. b, Western blot of proteins from wild type or EAT-D floral buds, probed with AP2 antibody. RbcL, large subunit of ribulose 1,5-bisphosphate carboxylase as loading control.

Figure 7. Identification of LAT-D. a, Location of the T-DNA insert in LAT-D, in between At2g28550 and At2g28560. The 4X 35S enhancers are approximately 5 kb from At2g28550. b, RT-PCR analysis of At2g28550 expression in wild type versus LAT-D plants.

Figure 8. EAT-D is epistatic to LAT-D. Genetic cross between EAT-D and LAT-D plants, with the resultant F1 plants shown, along with their flowering time (measured as rosette leaf number).

Figure 9. Loss-of-function At2g28550 (2-28550) and At5g60120 (6-60120) mutants. Location of T-DNA in each line is indicated, along with intron/exon structure.

Figure 10. Potential function of the miR172 miRNA family. a, Temporal expression of miR172a-2 and its relatives may cause temporal downregulation of

AP2 targets (e.g. At2g28550 and At5g60120), which may trigger flowering once the target proteins drop below a critical threshold (dotted line). b, Dicer cleavage at various positions may generate at least four distinct miRNAs from the miR172 family (indicated as a single hairpin with a miRNA consensus sequence). Sequences at the 5' and 3' ends of each miRNA are indicated, with the invariant middle 15 nt shown as ellipses. The putative targets recognized by the individual miRNAs are in parentheses below each.

Summary of the Invention

The invention provides methods and compositions useful in target sequence suppression and target sequence validation. The invention provides polynucleotide constructs useful for gene silencing, as well as cells, plants and seeds comprising the polynucleotides. The invention also provides a method for using microRNA to silence a target sequence.

Detailed Description

Recently discovered small RNAs play an important role in controlling gene expression. Regulation of many developmental processes including flowering is controlled by small RNAs. It is now possible to engineer changes in gene expression of plant genes by using transgenic constructs which produce small RNAs in the plant.

The invention provides methods and compositions useful for suppressing targeted sequences. The compositions can be employed in any type of plant cell, and in other cells which comprise the appropriate processing components (*e.g.*, RNA interference components), including invertebrate and vertebrate animal cells. The compositions and methods are based on an endogenous miRNA silencing process discovered in *Arabidopsis*, a similar strategy can be used to extend the number of compositions and the organisms in which the methods are used. The methods can be adapted to work in any eukaryotic cell system. Additionally, the compositions and methods described herein can be used in individual cells, cells or tissue in culture, or *in vivo* in organisms, or in organs or other portions of organisms.

The compositions selectively suppress the target sequence by encoding a miRNA having substantial complementarity to a region of the target sequence.

The miRNA is provided in a nucleic acid construct which, when transcribed into RNA, is predicted to form a hairpin structure which is processed by the cell to generate the miRNA, which then suppresses expression of the target sequence.

A nucleic acid construct is provided to encode the miRNA for any specific target sequence. Any miRNA can be inserted into the construct, such that the encoded miRNA selectively targets and suppresses the target sequence. The construct is modeled on the EAT (mir-172a) miRNA precursor from *Arabidopsis*.

A method for suppressing a target sequence is provided. The method employs the constructs above, in which a miRNA is designed to a region of the target sequence, and inserted into the construct. Upon introduction into a cell, the miRNA produced suppresses expression of the targeted sequence. The target sequence can be an endogenous plant sequence, or a heterologous transgene in the plant. The target gene may also be a gene from a plant pathogen, such as a pathogenic virus, nematode, insect, or mold or fungus.

A plant, cell, and seed comprising the construct and/or the miRNA is provided. Typically, the cell will be a cell from a plant, but other prokaryotic or eukaryotic cells are also contemplated, including but not limited to viral, bacterial, yeast, insect, nematode, or animal cells. Plant cells include cells from monocots and dicots. The invention also provides plants and seeds comprising the construct and/or the miRNA.

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxyl orientation, respectively. Numeric ranges recited within the specification are inclusive of the numbers defining the range and include each integer within the defined range. Amino acids may be referred to herein by either commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. Unless otherwise provided for, software, electrical, and electronics terms as used herein are as defined in The New IEEE Standard Dictionary of Electrical and Electronics Terms (5th edition, 1993). The terms defined below are more fully defined by reference to the specification as a whole.

As used herein, "nucleic acid construct" or "construct" refers to an isolated polynucleotide which is introduced into a host cell. This construct may comprise any combination of deoxyribonucleotides, ribonucleotides, and/or modified nucleotides. The construct may be transcribed to form an RNA, wherein the RNA may be capable of forming a double-stranded RNA and/or hairpin structure. This construct may be expressed in the cell, or isolated or synthetically produced. The construct may further comprise a promoter, or other sequences which facilitate manipulation or expression of the construct.

As used here "suppression" or "silencing" or "inhibition" are used interchangeably to denote the down-regulation of the expression of the product of a target sequence relative to its normal expression level in a wild type organism. Suppression includes expression that is decreased by about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% relative to the wild type expression level.

As used herein, "encodes" or "encoding" refers to a DNA sequence which can be processed to generate an RNA and/or polypeptide.

As used herein, "expression" or "expressing" refers to the generation of an RNA transcript from an introduced construct, an endogenous DNA sequence, or a stably incorporated heterologous DNA sequence. The term may also refer to a polypeptide produced from an mRNA generated from any of the above DNA precursors.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or is synthetically designed, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

By "host cell" is meant a cell which contains an introduced nucleic acid construct and supports the replication and/or expression of the construct. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as fungi, yeast, insect, amphibian, nematode, or mammalian cells. Alternatively, the host cells are monocotyledonous or dicotyledonous plant cells. An example of a monocotyledonous host cell is a maize host cell.

The term "introduced" means providing a nucleic acid or protein into a cell. Introduced includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell, and includes reference to the transient provision of a nucleic acid or protein to the cell. Introduced includes reference to stable or transient transformation methods, as well as sexually crossing.

The term "isolated" refers to material, such as a nucleic acid or a protein, which is: (1) substantially or essentially free from components which normally accompany or interact with the material as found in its naturally occurring environment or (2) if the material is in its natural environment, the material has been altered by deliberate human intervention to a composition and/or placed at a locus in the cell other than the locus native to the material.

As used herein, "miRNA" refers to an oligoribonucleic acid, which suppresses expression of a polynucleotide comprising the target sequence transcript. A "miRNA precursor" refers to a larger polynucleotide which is processed to produce a mature miRNA, and includes a DNA which encodes an RNA precursor, and an RNA transcript comprising the miRNA. A "mature miRNA" refers to the miRNA generated from the processing of a miRNA precursor. A "miRNA template" is an oligonucleotide region, or regions, in a nucleic acid construct which encodes the miRNA. The "backside" region of a miRNA is a portion of a polynucleotide construct which is substantially complementary to the miRNA template and is predicted to base pair with the miRNA template. The miRNA template and backside may form a double-stranded polynucleotide, including a hairpin structure.

As used herein, the phrases "target sequence" and "sequence of interest" are used interchangeably. Target sequence is used to mean the nucleic acid sequence that is selected for suppression of expression, and is not limited to polynucleotides encoding polypeptides. The target sequence comprises a sequence that is substantially or completely complementary to the miRNA. The target sequence can be RNA or DNA, and may also refer to a polynucleotide comprising the target sequence.

As used herein, "nucleic acid" means a polynucleotide and includes single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. Nucleic acids may also include fragments and modified nucleotides.

By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism or of a tissue from that organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such as Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology,* Vol. 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook et al., *Molecular Cloning - A Laboratory Manual,* 2nd ed., Vol. 1-3 (1989); and *Current Protocols in Molecular Biology*, F.M. Ausubel et al., Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994).

As used herein "operably linked" includes reference to a functional linkage of at least two sequences. Operably linked includes linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence.

As used herein, "plant" includes plants and plant parts including but not limited to plant cells, plant tissue such as leaves, stems, roots, flowers, and seeds.

As used herein, "polypeptide" means proteins, protein fragments, modified proteins, amino acid sequences and synthetic amino acid sequences. The polypeptide can be glycosylated or not.

As used herein, "promoter" includes reference to a region of DNA that is involved in recognition and binding of an RNA polymerase and other proteins to initiate transcription.

The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (*e.g.*, at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, or 90% sequence identity, up to and including 100% sequence identity (*i.e.*, fully complementary) with each other.

The term "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will selectively hybridize to its target sequence. Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the

hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984): $T_m = 81.5\ °C + 16.6\ (\log M) + 0.41\ (\%GC) - 0.61\ (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH.

However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, Chapter 2, Ausubel et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Hybridization and/or wash conditions can be applied for at least 10, 30, 60, 90, 120, or 240 minutes.

As used herein, "transgenic" includes reference to a plant or a cell which comprises a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. Transgenic is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

As used herein, "vector" includes reference to a nucleic acid used in introduction of a polynucleotide of the invention into a host cell. Expression vectors permit transcription of a nucleic acid inserted therein.

Polynucleotide sequences may have substantial identity, substantial homology, or substantial complementarity to the selected region of the target gene. As used herein "substantial identity" and "substantial homology" indicate sequences that have sequence identity or homology to each other. Generally, sequences that are substantially identical or substantially homologous will have about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity wherein the percent sequence identity is based on the entire sequence and is determined by GAP alignment using default parameters (GCG, GAP version 10, Accelrys, San Diego, CA). GAP uses the algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443-453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of sequence gaps. Sequences which have 100% identity are identical. "Substantial complementarity" refers to sequences that are complementary to each other, and are able to base pair with each other. In describing complementary sequences, if all the nucleotides in the first sequence will base pair to the second sequence, these sequences are fully complementary.

Through a forward genetics approach, a microRNA that confers a developmental phenotype in *Arabidopsis* was identified. This miRNA, miR172a-2 (Park *et al.*, *Curr. Biol.* 12:1484-1495 2002), causes early flowering and defects in floral organ identity when overexpressed. The predicted target of miR172a-2 is a small subfamily of APETALA2-like transcription factors (Okamuro *et al.* 1997). Overexpression of miR172a-2 downregulates at least one member of this family. In addition, overexpression of one of the AP2-like target genes, At2g28550, causes late flowering. This result, in conjunction with loss-of-function analyses of At2g28550 and another target gene, At5g60120, indicates that at least some of the AP2-like genes targeted by miR172a-2 normally function as floral repressors. The EAT-D line overexpressing miR172-a2 has a wild-type response to photoperiod. The genomic region encoding the miRNA was also identified (SEQ ID NO: 1) and used to produce a cassette into which other miRNAs to target sequences can be inserted (SEQ ID NO: 3), and to produce an expression vector (SEQ ID NO: 44) useful for cloning the cassettes and expressing the miRNA. The expression vector comprises the 1.4kb region encoding the miRNA. Expression of this region is processed in the cell to produce the miRNA which suppresses

expression of the target gene. Alternatively, the miRNA may be synthetically produced and introduced to the cell directly.

In one embodiment, there is provided a method for the suppression of a target sequence comprising introducing into a cell a nucleic acid construct encoding a miRNA substantially complementary to the target. In some embodiments the miRNA comprises about 10-200 nucleotides, about 10-15, 15-20, 19, 20, 21, 22, 23, 24, 25, 26, 27, 25-30, 30-50, 50-100, 100-150, or about 150-200 nucleotides. In some embodiments the nucleic acid construct encodes the miRNA. In some embodiments the nucleic acid construct encodes a polynucleotide precursor which may form a double-stranded RNA, or hairpin structure comprising the miRNA. In some embodiments, nucleotides 39-59 and 107-127 of SEQ ID NO: 3 are replaced by the backside of the miRNA template and the miRNA template respectively. In some embodiments, this new sequence replaces the equivalent region of SEQ ID NO: 1. In further embodiments, this new sequence replaces the equivalent region of SEQ ID NO: 44.

In some embodiments, the nucleic acid construct comprises a modified endogenous plant miRNA precursor, wherein the precursor has been modified to replace the endogenous miRNA encoding regions with sequences designed to produce a miRNA directed to the target sequence. In some embodiments the miRNA precursor template is a miR172a miRNA precursor. In some embodiments, the miR172a precursor is from a dicot or a monocot. In some embodiments the miR172a precursor is from *Arabidopsis thaliana*, tomato, soybean, rice, or corn. In some embodiments the miRNA precursor is SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 44.

In another embodiment the method comprises:

A method of inhibiting expression of a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide, wherein the polynucleotide comprises in the following order:

(i) at least about 20 contiguous nucleotides in the region of nucleotides 1-38 of SEQ ID NO: 3,

(ii) a first oligonucleotide of 10 to about 50 contiguous nucleotides, wherein the first oligonucleotide is substantially complementary to a second oligonucleotide

(iii) at least about 20 contiguous nucleotides in the region of nucleotides 60-106 of SEQ ID NO: 3,

(iv) the second oligonucleotide of about 10 to about 50 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and the second oligonucleotide is substantially complementary to the target sequence, and

(v) at least about 20 contiguous nucleotides in the region of nucleotides 128-159 of SEQ ID NO:3;

wherein the polynucleotide encodes an RNA precursor capable of forming a hairpin, and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

In another embodiment the method comprises:

A method of inhibiting expression of a gene comprising a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide, wherein the polynucleotide comprises in the following order:

(i) nucleotides 1-38 of SEQ ID NO: 3,

(ii) a first oligonucleotide of 21 contiguous nucleotides, wherein the first oligonucleotide is substantially complementary to a second oligonucleotide,

(iii) nucleotides 60-106 of SEQ ID NO: 3,

(iv) the second oligonucleotide of 21 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and wherein the second oligonucleotide is substantially complementary to the target sequence, and

(v) nucleotides 128-159 of SEQ ID NO:3;

wherein polynucleotide encodes an RNA precursor capable of forming a hairpin, and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

In another embodiment, the method comprises selecting a target sequence of a gene, and designing a nucleic acid construct comprising polynucleotide encoding a miRNA substantially complementary to the target sequence. In some embodiments, the target sequence is selected from any region of the gene. In

some embodiments, the target sequence is selected from an untranslated region. In some embodiments, the target sequence is selected from a coding region of the gene. In some embodiments, the target sequence is selected from a region about 50 to about 200 nucleotides upstream from the stop codon, including regions from about 50-75, 75-100, 100-125, 125-150, or 150-200 upstream from the stop codon. In further embodiments, the target sequence and/or the miRNA is based on the polynucleotides and process of EAT suppression of Apetela2-like genes in *Arabidopsis thaliana*. In some embodiments, nucleotides 39-59 and 107-127 of SEQ ID NO: 3 are replaced by the backside of the miRNA template (first oligonucleotide) and the miRNA template (second oligonucleotide) respectively. In some embodiments, this new sequence replaces the equivalent region of SEQ ID NO: 1. In further embodiments, this new sequence replaces the equivalent region of SEQ ID NO: 44.

In some embodiments, the miRNA template, (*i.e.* the polynucleotide encoding the miRNA), and thereby the miRNA, may comprise some mismatches relative to the target sequence. In some embodiments the miRNA template has ≥ 1 nucleotide mismatch as compared to the target sequence, for example, the miRNA template can have 1, 2, 3, 4, 5, or more mismatches as compared to the target sequence. This degree of mismatch may also be described by determining the percent identity of the miRNA template to the complement of the target sequence. For example, the miRNA template may have a percent identity including about at least 70%, 75%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% as compared to the complement of the target sequence.

In some embodiments, the miRNA template, (*i.e.* the polynucleotide encoding the miRNA) and thereby the miRNA, may comprise some mismatches relative to the miRNA backside. In some embodiments the miRNA template has ≥ 1 nucleotide mismatch as compared to the miRNA backside, for example, the miRNA template can have 1, 2, 3, 4, 5, or more mismatches as compared to the miRNA backside. This degree of mismatch may also be described by determining the percent identity of the miRNA template to the complement of the miRNA backside. For example, the miRNA template may have a percent identity including about at least 70%, 75%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%,

85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% as compared to the complement of the miRNA backside.

In some embodiments, the target sequence is selected from a plant pathogen. Plants or cells comprising a miRNA directed to the target sequence of the pathogen are expected to have decreased sensitivity and/or increased resistance to the pathogen. In some embodiments, the miRNA is encoded by a nucleic acid construct further comprising an operably linked promoter. In some embodiments, the promoter is a pathogen-inducible promoter.

In another embodiment, the method comprises replacing the miRNA encoding sequence in the polynucleotide of SEQ ID NO: 3 with a sequence encoding a miRNA substantially complementary to the target region of the target gene.

In another embodiment a method is provided comprising a method of inhibiting expression of a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide encoding a modified plant miRNA precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide encodes a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin; and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

In another embodiment a method is provided comprising a method of inhibiting expression of a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide encoding a modified plant miR172 miRNA precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide encodes a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin; and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

In another embodiment a method is provided comprising a method of inhibiting expression of a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide encoding a modified *Arabidopsis* miR172 miRNA precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide encodes a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin; and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

In another embodiment a method is provided comprising a method of inhibiting expression of a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide encoding a modified corn miR172 miRNA precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide encodes a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin; and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

In another embodiment, there is provided a nucleic acid construct for suppressing a target sequence. The nucleic acid construct encodes a miRNA substantially complementary to the target. In some embodiments, the nucleic acid construct further comprises a promoter operably linked to the polynucleotide encoding the miRNA. In some embodiments, the nucleic acid construct lacking a promoter is designed and introduced in such a way that it becomes operably linked to a promoter upon integration in the host genome. In some embodiments, the nucleic acid construct is integrated using recombination, including site-specific

recombination. See, for example, WO 99/25821, herein incorporated by reference. In some embodiments, the nucleic acid construct is an RNA. In some embodiments, the nucleic acid construct comprises at least one recombination site, including site-specific recombination sites. In some embodiments the nucleic acid construct comprises at least one recombination site in order to facilitate integration, modification, or cloning of the construct. In some embodiments the nucleic acid construct comprises two site-specific recombination sites flanking the miRNA precursor. In some embodiments the site-specific recombination sites include FRT sites, lox sites, or att sites, including attB, attL, attP or attR sites. See, for example, WO 99/25821, and U.S. Patents 5,888,732, 6,143,557, 6,171,861, 6,270,969, and 6,277,608, herein incorporated by reference.

In some embodiments, the nucleic acid construct comprises a modified endogenous plant miRNA precursor, wherein the precursor has been modified to replace the miRNA encoding region with a sequence designed to produce a miRNA directed to the target sequence. In some embodiments the miRNA precursor template is a miR172a miRNA precursor. In some embodiments, the miR172a precursor is from a dicot or a monocot. In some embodiments the miR172a precursor is from *Arabidopsis thaliana*, tomato, soybean, rice, or corn. In some embodiments the miRNA precursor is SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 44.

In another embodiment, the nucleic acid construct comprises an isolated polynucleotide comprising a polynucleotide which encodes a modified plant miRNA precursor, the modified precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide i is substantially complementary to the second oligonucleotide, and the second oligonucleotide comprises a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin.

In another embodiment, the nucleic acid construct comprises an isolated polynucleotide comprising a polynucleotide which encodes a modified plant miR172 miRNA precursor, the modified precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide

comprises a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin.

In another embodiment, the nucleic acid construct comprises an isolated polynucleotide comprising a polynucleotide which encodes a modified Arabidopsis miR172 miRNA precursor, the modified precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide comprises a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin.

In another embodiment, the nucleic acid construct comprises an isolated polynucleotide comprising a polynucleotide which encodes a modified corn miR172 miRNA precursor, the modified precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide comprises a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin.

In some embodiments the miRNA comprises about 10-200 nucleotides, about 10-15, 15-20, 19, 20, 21, 22, 23, 24, 25, 26, 27, 25-30, 30-50, 50-100, 100-150, or about 150-200 nucleotides. In some embodiments the nucleic acid construct encodes the miRNA. In some embodiments the nucleic acid construct encodes a polynucleotide precursor which may form a double-stranded RNA, or hairpin structure comprising the miRNA. In some embodiments, nucleotides 39-59 and/or 107-127 of SEQ ID NO: 3 are replaced by the backside of the miRNA template and the miRNA template respectively. In some embodiments, this new sequence replaces the equivalent region of SEQ ID NO: 1. In further embodiments, this new sequence replaces the equivalent region of SEQ ID NO: 44. In some embodiments, the target region is selected from any region of the target sequence. In some embodiments, the target region is selected from a untranslated region. In some embodiments, the target region is selected from a coding region of the target sequence. In some embodiments, the target region is selected from a region about 50 to about 200 nucleotides upstream from the stop codon, including regions from about 50-75, 75-100, 100-125, 125-150, or 150-200

upstream from the stop codon. In further embodiments, the target region and/or the miRNA is based on the polynucleotides and process of EAT suppression of Apetela2-like sequences in *Arabidopsis thaliana*.

In another embodiment the nucleic acid construct comprises an isolated polynucleotide comprising in the following order at least 20 contiguous nucleotides in the region from nucleotides 1-38 of SEQ ID NO: 3, a first oligonucleotide of about 10 to about 50 contiguous nucleotides, wherein the first oligonucleotide is substantially complementary to a second oligonucleotide, at least about 20 contiguous nucleotides in the region from nucleotides 60-106 of SEQ ID NO: 3, a second oligonucleotide of about 10 to about 50 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and the second oligonucleotide is substantially complementary to the target sequence, and at least about 20 contiguous nucleotides in the region from nucleotides 128-159 of SEQ ID NO: 3, wherein the polynucleotide encodes an RNA precursor capable of forming a hairpin structure.

In another embodiment the nucleic acid construct comprises an isolated polynucleotide comprising in the following order nucleotides 1-38 of SEQ ID NO: 3, a first oligonucleotide of 21 contiguous nucleotides, wherein the first oligonucleotide is substantially complementary to a second oligonucleotide, nucleotides 60-106 of SEQ ID NO: 3, a second oligonucleotide of 21 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and the second oligonucleotide is substantially complementary to the target sequence, and nucleotides 128-159 of SEQ ID NO: 3, wherein the polynucleotide encodes an RNA precursor capable of forming a hairpin.

In some embodiments there are provided cells, plants, and seeds comprising the introduced polynucleotides, and/or produced by the methods of the invention. The cells include prokaryotic and eukaryotic cells, including but not limited to bacteria, yeast, fungi, viral, invertebrate, vertebrate, and plant cells. Plants, plant cells, and seeds of the invention include gynosperms, monocots and dicots, including but not limited to, for example, rice, wheat, oats, barley, millet, sorghum, soy, sunflower, safflower, canola, alfalfa, cotton, *Arabidopsis*, and tobacco.

In some embodiments, the cells, plants, and/or seeds comprise a nucleic acid construct comprising a modified plant miRNA precursor, wherein the

precursor has been modified to replace the endogenous miRNA encoding regions with sequences designed to produce a miRNA directed to the target sequence. In some embodiments the miRNA precursor template is a miR172a miRNA precursor. In some embodiments, the miR172a precursor is from a dicot or a monocot. In some embodiments the miR172a precursor is from *Arabidopsis thaliana*, tomato, soybean, rice, or corn. In some embodiments the miRNA precursor is SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 44. In some embodiments the miRNA precursor is encoded by SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 44. In some embodiments, the nucleic acid construct comprises at least one recombination site, including site-specific recombination sites. In some embodiments the nucleic acid construct comprises at least one recombination site in order to facilitate modification or cloning of the construct. In some embodiments the nucleic acid construct comprises two site-specific recombination sites flanking the miRNA precursor. In some embodiments the site-specific recombination sites include FRT sites, lox sites, or att sites, including attB, attL, attP or attR sites. See, for example, WO 99/25821, and U.S. Patents 5,888,732, 6,143,557, 6,171,861, 6,270,969, and 6,277,608, herein incorporated by reference.

The present invention concerns methods and compositions useful in suppression of a target sequence and/or validation of function. The invention also relates to a method for using microRNA (miRNA) mediated RNA interference (RNAi) to silence or suppress a target sequence to evaluate function, or to validate a target sequence for phenotypic effect and/or trait development. Specifically, the invention relates to constructs comprising small nucleic acid molecules, miRNAs, capable of inducing silencing, and methods of using these miRNAs to selectively silence target sequences.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, *Nature* 391:806 1998). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing (PTGS) or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.*, *Trends Genet.* 15:358

1999). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA of viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.*, *Nature* 409:363 2001). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Elbashir *et al.*, *Genes Dev.* 15:188 2001). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science* 293:834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementarity to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir *et al.*, *Genes Dev.* 15:188 2001). In addition, RNA interference can also involve small RNA (*e.g.*, microRNA, or miRNA) mediated gene silencing, presumably through cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see, *e.g.*, Allshire, *Science* 297:1818-1819 2002; Volpe *et al.*, *Science* 297:1833-1837 2002; Jenuwein, *Science* 297:2215-2218 2002; and Hall *et al.*, *Science* 297:2232-2237 2002). As such, miRNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional or post-transcriptional level.

RNAi has been studied in a variety of systems. Fire *et al.* (*Nature* 391:806 1998) were the first to observe RNAi in *C. elegans*. Wianny and Goetz (*Nature Cell Biol.* 2:70 1999) describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.* (*Nature* 404:293 2000) describe RNAi in *Drosophila* cells

transfected with dsRNA. Elbashir *et al.*, (*Nature* 411:494 2001) describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells.

Small RNAs play an important role in controlling gene expression. Regulation of many developmental processes, including flowering, is controlled by small RNAs. It is now possible to engineer changes in gene expression of plant genes by using transgenic constructs which produce small RNAs in the plant.

Small RNAs appear to function by base-pairing to complementary RNA or DNA target sequences. When bound to RNA, small RNAs trigger either RNA cleavage or translational inhibition of the target sequence. When bound to DNA target sequences, it is thought that small RNAs can mediate DNA methylation of the target sequence. The consequence of these events, regardless of the specific mechanism, is that gene expression is inhibited.

It is thought that sequence complementarity between small RNAs and their RNA targets helps to determine which mechanism, RNA cleavage or translational inhibition, is employed. It is believed that siRNAs, which are perfectly complementary with their targets, work by RNA cleavage. Some miRNAs have perfect or near-perfect complementarity with their targets, and RNA cleavage has been demonstrated for at least a few of these miRNAs. Other miRNAs have several mismatches with their targets, and apparently inhibit their targets at the translational level. Again, without being held to a particular theory on the mechanism of action, a general rule is emerging that perfect or near-perfect complementarity favors RNA cleavage, whereas translational inhibition is favored when the miRNA/target duplex contains many mismatches. The apparent exception to this is microRNA 172 (miR172) in plants. One of the targets of miR172 is APETALA2 (AP2), and although miR172 shares near-perfect complementarity with AP2 it appears to cause translational inhibition of AP2 rather than RNA cleavage.

MicroRNAs (miRNAs) are noncoding RNAs of about 19 to about 24 nucleotides (nt) in length that have been identified in both animals and plants (Lagos-Quintana *et al.*, *Science* 294:853-858 2001, Lagos-Quintana *et al.*, *Curr. Biol.* 12:735-739 2002; Lau *et al.*, *Science* 294:858-862 2001; Lee and Ambros, *Science* 294:862-864 2001; Llave *et al.*, *Plant Cell* 14:1605-1619 2002; Mourelatos *et al.*, *Genes. Dev.* 16:720-728 2002; Park *et al.*, *Curr. Biol.* 12:1484-

1495 2002; Reinhart *et al.*, *Genes. Dev.* 16:1616-1626 2002). They are processed from longer precursor transcripts that range in size from approximately 70 to 200 nt, and these precursor transcripts have the ability to form stable hairpin structures. In animals, the enzyme involved in processing miRNA precursors is called Dicer, an RNAse III-like protein (Grishok *et al.*, *Cell* 106:23-34 2001; Hutvagner *et al.*, *Science* 293:834-838 2001; Ketting *et al.*, *Genes. Dev.* 15:2654-2659 2001). Plants also have a Dicer-like enzyme, DCL1 (previously named CARPEL FACTORY/SHORT INTEGUMENTS1/ SUSPENSOR1), and recent evidence indicates that it, like Dicer, is involved in processing the hairpin precursors to generate mature miRNAs (Park *et al.*, *Curr. Biol.* 12:1484-1495 2002; Reinhart *et al.*, *Genes. Dev.* 16:1616-1626 2002). Furthermore, it is becoming clear from recent work that at least some miRNA hairpin precursors originate as longer polyadenylated transcripts, and several different miRNAs and associated hairpins can be present in a single transcript (Lagos-Quintana *et al.*, *Science* 294:853-858 2001; Lee *et al.*, *EMBO J* 21:4663-4670 2002). Recent work has also examined the selection of the miRNA strand from the dsRNA product arising from processing of the hairpin by DICER (Schwartz, et al. 2003 Cell 115:199-208). It appears that the stability (*i.e.* G:C vs. A:U content, and/or mismatches) of the two ends of the processed dsRNA affects the strand selection, with the low stability end being easier to unwind by a helicase activity. The 5' end strand at the low stability end is incorporated into the RISC complex, while the other strand is degraded.

In animals, there is direct evidence indicating a role for specific miRNAs in development. The lin-4 and let-7 miRNAs in *C. elegans* have been found to control temporal development, based on the phenotypes generated when the genes producing the lin-4 and let-7 miRNAs are mutated (Lee *et al.*, *Cell* 75:843-854 1993; Reinhart *et al.*, *Nature* 403-901-906 2000). In addition, both miRNAs display a temporal expression pattern consistent with their roles in developmental timing. Other animal miRNAs display developmentally regulated patterns of expression, both temporal and tissue-specific (Lagos-Quintana *et al.*, *Science* 294:853-853 2001, Lagos-Quintana *et al.*, *Curr. Biol.* 12:735-739 2002; Lau *et al.*, *Science* 294:858-862 2001; Lee and Ambros, *Science* 294:862-864 2001), leading to the hypothesis that miRNAs may, in many cases, be involved in the regulation of important developmental processes. Likewise, in plants, the differential

expression patterns of many miRNAs suggests a role in development (Llave *et al.*, *Plant Cell* 14:1605-1619 2002; Park *et al.*, *Curr. Biol.* 12:1484-1495 2002; Reinhart *et al.*, *Genes. Dev.* 16:1616-1626 2002). However, a developmental role for miRNAs has not been directly proven in plants, because to date there has been no report of a developmental phenotype associated with a specific plant miRNA.

MicroRNAs appear to regulate target genes by binding to complementary sequences located in the transcripts produced by these genes. In the case of lin-4 and let-7, the target sites are located in the 3' UTRs of the target mRNAs (Lee *et al.*, *Cell* 75:843-854 1993; Wightman *et al.*, *Cell* 75:855-862 1993; Reinhart *et al.*, *Nature* 403:901-906 2000; Slack *et al.*, *Mol. Cell* 5:659-669 2000), and there are several mismatches between the lin-4 and let-7 miRNAs and their target sites. Binding of the lin-4 or let-7 miRNA appears to cause downregulation of steady-state levels of the protein encoded by the target mRNA without affecting the transcript itself (Olsen and Ambros, *Dev. Biol.* 216:671-680 1999). On the other hand, recent evidence suggests that miRNAs can, in some cases, cause specific RNA cleavage of the target transcript within the target site, and this cleavage step appears to require 100% complementarity between the miRNA and the target transcript (Hutvagner and Zamore, *Science* 297:2056-2060 2002; Llave *et al.*, *Plant Cell* 14:1605-1619 2002). It seems likely that miRNAs can enter at least two pathways of target gene regulation: Protein downregulation when target complementarity is <100%, and RNA cleavage when target complementarity is 100%. MicroRNAs entering the RNA cleavage pathway are analogous to the 21-25 nt short interfering RNAs (siRNAs) generated during RNA interference (RNAi) in animals and posttranscriptional gene silencing (PTGS) in plants (Hamilton and Baulcombe 1999; Hammond *et al.*, 2000; Zamore *et al.*, 2000; Elbashir *et al.*, 2001), and likely are incorporated into an RNA-induced silencing complex (RISC) that is similar or identical to that seen for RNAi.

Identifying the targets of miRNAs with bioinformatics has not been successful in animals, and this is probably due to the fact that animal miRNAs have a low degree of complementarity with their targets. On the other hand, bioinformatic approaches have been successfully used to predict targets for plant miRNAs (Llave *et al.*, *Plant Cell* 14:1605-1619 2002; Park *et al.*, *Curr. Biol.* 12:1484-1495 2002; Rhoades *et al.*, *Cell* 110:513-520 2002), and thus it appears

that plant miRNAs have higher overall complementarity with their putative targets than do animal miRNAs. Most of these predicted target transcripts of plant miRNAs encode members of transcription factor families implicated in plant developmental patterning or cell differentiation. Nonetheless, biological function has not been directly demonstrated for any plant miRNA. Although Llave *et al.* (*Science* 297:2053-2056 2002) have shown that a transcript for a SCARECROW-like transcription factor is a target of the *Arabidopsis* miRNA mir171, these studies were performed in a heterologous species and no plant phenotype associated with mir171 was reported.

The methods provided can be practiced in any organism in which a method of transformation is available, and for which there is at least some sequence information for the target sequence, or for a region flanking the target sequence of interest. It is also understood that two or more sequences could be targeted by sequential transformation, co-transformation with more than one targeting vector, or the construction of a DNA construct comprising more than one miRNA sequence. The methods of the invention may also be implemented by a combinatorial nucleic acid library construction in order to generate a library of miRNAs directed to random target sequences. The library of miRNAs could be used for high-throughput screening for gene function validation.

General categories of sequences of interest include, for example, those genes involved in regulation or information, such as zinc fingers, transcription factors, homeotic genes, or cell cycle and cell death modulators, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins.

Target sequences further include coding regions and non-coding regions such as promoters, enhancers, terminators, introns and the like, which may be modified in order to alter the expression of a gene of interest. For example, an intron sequence can be added to the 5' region to increase the amount of mature message that accumulates (see for example Buchman and Berg, *Mol. Cell Biol.* 8:4395-4405 (1988); and Callis *et al.*, *Genes Dev.* 1:1183-1200 (1987)).

The target sequence may be an endogenous sequence, or may be an introduced heterologous sequence, or transgene. For example, the methods may be used to alter the regulation or expression of a transgene, or to remove a transgene or other introduced sequence such as an introduced site-specific

recombination site. The target sequence may also be a sequence from a pathogen, for example, the target sequence may be from a plant pathogen such as a virus, a mold or fungus, an insect, or a nematode. A miRNA could be expressed in a plant which, upon infection or infestation, would target the pathogen and confer some degree of resistance to the plant.

In plants, other categories of target sequences include genes affecting agronomic traits, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and commercial products. Genes of interest also included those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting, for example, kernel size, sucrose loading, and the like. The quality of grain is reflected in traits such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids, and levels of cellulose. For example, genes of the phytic acid biosynthetic pathway could be suppressed to generate a high available phosphorous phenotype. See, for example, phytic acid biosynthetic enzymes including inositol polyphosphate kinase-2 polynucleotides, disclosed in WO 02/059324, inositol 1,3,4-trisphosphate 5/6-kinase polynucleotides, disclosed in WO 03/027243, and myo-inositol 1-phosphate synthase and other phytate biosynthetic polynucleotides, disclosed in WO 99/05298, all of which are herein incorporated by reference. Genes in the lignification pathway could be suppressed to enhance digestibility or energy availability. Genes affecting cell cycle or cell death could be suppressed to affect growth or stress response. Genes affecting DNA repair and/or recombination could be suppressed to increase genetic variability. Genes affecting flowering time could be suppressed, as well as genes affecting fertility. Any target sequence could be suppressed in order to evaluate or confirm its role in a particular trait or phenotype, or to dissect a molecular, regulatory, biochemical, or proteomic pathway or network.

A number of promoters can be used, these promoters can be selected based on the desired outcome. It is recognized that different applications will be enhanced by the use of different promoters in plant expression cassettes to modulate the timing, location and/or level of expression of the miRNA. Such plant expression cassettes may also contain, if desired, a promoter regulatory region (*e.g.*, one conferring inducible, constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation

start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Constitutive, tissue-preferred or inducible promoters can be employed. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, the ubiquitin 1 promoter, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (U.S. Patent No. 5,683,439), the Nos promoter, the pEmu promoter, the rubisco promoter, the GRP1-8 promoter and other transcription initiation regions from various plant genes known to those of skill. If low level expression is desired, weak promoter(s) may be used. Weak constitutive promoters include, for example, the core promoter of the Rsyn7 promoter (WO 99/43838 and U.S. Patent No. 6,072,050), the core 35S CaMV promoter, and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142. See also, U.S. Patent No. 6,177,611, herein incorporated by reference.

Examples of inducible promoters are the Adh1 promoter which is inducible by hypoxia or cold stress, the Hsp70 promoter which is inducible by heat stress, the PPDK promoter and the pepcarboxylase promoter which are both inducible by light. Also useful are promoters which are chemically inducible, such as the In2-2 promoter which is safener induced (U.S. patent 5,364,780), the ERE promoter which is estrogen induced, and the Axig1 promoter which is auxin induced and tapetum specific but also active in callus (PCT US01/22169).

Examples of promoters under developmental control include promoters that initiate transcription preferentially in certain tissues, such as leaves, roots, fruit, seeds, or flowers. An exemplary promoter is the anther specific promoter 5126 (U.S. Patent Nos. 5,689,049 and 5,689,051). Examples of seed-preferred promoters include, but are not limited to, 27 kD gamma zein promoter and waxy promoter, Boronat, A. *et al.* (1986) *Plant Sci.* 47:95-102; Reina, M. *et al. Nucl. Acids Res.* 18(21):6426; and Kloesgen, R.B. *et al.* (1986) *Mol. Gen. Genet.* 203:237-244. Promoters that express in the embryo, pericarp, and endosperm are disclosed in US patent 6,225,529 and PCT publication WO 00/12733. The disclosures each of these are incorporated herein by reference in their entirety.

In some embodiments it will be beneficial to express the gene from an inducible promoter, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; *e.g.*, PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. See also WO 99/43819, herein incorporated by reference.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200).

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the polynucleotides. Such wound-inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996) *Nature Biotech.* 14:494-498); wun1 and wun2, U.S. Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); systemin (McGurl *et al.* (1992) *Science* 225:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol.* 22:783-792; Eckelkamp *et al.* (1993) *FEBS Lett.* 323:73-76); MPI gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like, herein incorporated by reference.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a

chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced expression of a sequence of interest within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-preferred promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590. In addition, the promoters of cab and ribisco can also be used. See, for example, Simpson *et al.* (1958) *EMBO J* 4:2723-2729 and Timko *et al.* (1988) *Nature* 318:57-58.

Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species.

See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2):343-350). The TR1' gene, fused to nptII (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179. The phaseolin gene (Murai *et al.* (1983) *Science* 23:476-482 and Sengopta-Gopalen *et al.* (1988) *PNAS* 82:3320-3324.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.*, monocot or dicot, targeted for transformation. Suitable methods of introducing the DNA construct include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334; and U.S. Patent No. 6,300,543), sexual crossing, electroporation

(Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606), Agrobacterium-mediated transformation (Townsend *et al.*, U.S. Pat No. 5,563,055; and U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney *et al.*, U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature* (London) 311:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman et al. (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); and U.S. Patent No. 5,736,369 (meristem transformation), all of which are herein incorporated by reference.

The nucleotide constructs may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the invention within a viral DNA or RNA molecule. Further, it is recognized that useful promoters encompass promoters

utilized for transcription by viral RNA polymerases. Methods for introducing nucleotide constructs into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931; herein incorporated by reference.

In some embodiments, transient expression may be desired. In those cases, standard transient transformation techniques may be used. Such methods include, but are not limited to viral transformation methods, and microinjection of DNA or RNA, as well other methods well known in the art.

The cells from the plants that have stably incorporated the nucleotide sequence may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic imparted by the nucleotide sequence of interest and/or the genetic markers contained within the target site or transfer cassette. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

Initial identification and selection of cells and/or plants comprising the DNA constructs may be facilitated by the use of marker genes. Gene targeting can be performed without selection if there is a sensitive method for identifying recombinants, for example if the targeted gene modification can be easily detected by PCR analysis, or if it results in a certain phenotype. However, in most cases, identification of gene targeting events will be facilitated by the use of markers. Useful markers include positive and negative selectable markers as well as markers that facilitate screening, such as visual markers. Selectable markers include genes carrying resistance to an antibiotic such as spectinomycin (*e.g.* the aada gene, Svab *et al.* 1990 *Plant Mol. Biol.* 14:197), streptomycin (*e.g.*, aada, or SPT, Svab *et al.* 1990 *Plant Mol. Biol.* 14:197; Jones *et al.* 1987 *Mol. Gen. Genet.* 210:86), kanamycin (*e.g.*, nptII, Fraley *et al.* 1983 *PNAS* 80:4803), hygromycin (*e.g.*, HPT, Vanden Elzen *et al.* 1985 *Plant Mol. Biol.* 5:299), gentamycin (Hayford *et al.* 1988 *Plant Physiol.* 86:1216), phleomycin, zeocin, or bleomycin (Hille *et al.*

1986 *Plant Mol. Biol.* 7:171), or resistance to a herbicide such as phosphinothricin (bar gene), or sulfonylurea (acetolactate synthase (ALS)) (Charest *et al.* (1990) *Plant Cell Rep.* 8:643), genes that fulfill a growth requirement on an incomplete media such as HIS3, LEU2, URA3, LYS2, and TRP1 genes in yeast, and other such genes known in the art. Negative selectable markers include cytosine deaminase (codA) (Stougaard 1993 *Plant J.* 3:755-761), tms2 (DePicker *et al.* 1988 *Plant Cell Rep.* 7:63-66), nitrate reductase (Nussame *et al.* 1991 *Plant J.* 1:267-274), SU1 (O'Keefe *et al.* 1994 *Plant Physiol.* 105:473-482), aux-2 from the Ti plasmid of Agrobacterium, and thymidine kinase. Screenable markers include fluorescent proteins such as green fluorescent protein (GFP) (Chalfie *et al.*, 1994 *Science* 263:802; US 6,146,826; US 5,491,084; and WO 97/41228), reporter enzymes such as β-glucuronidase (GUS) (Jefferson R.A. 1987 *Plant Mol. Biol. Rep.* 5:387; US 5,599,670; and US 5,432,081), β-galactosidase (lacZ), alkaline phosphatase (AP), glutathione S-transferase (GST) and luciferase (US 5,674,713; and Ow *et al.* 1986 *Science* 234(4778):856-859), visual markers like anthocyanins such as CRC (Ludwig *et al.* (1990) *Science* 247(4841):449-450) R gene family (*e.g.* Lc, P, S), A, C, R-nj, body and/or eye color genes in *Drosophila*, coat color genes in mammalian systems, and others known in the art.

One or more markers may be used in order to select and screen for gene targeting events. One common strategy for gene disruption involves using a target modifying polynucleotide in which the target is disrupted by a promoterless selectable marker. Since the selectable marker lacks a promoter, random integration events are unlikely to lead to transcription of the gene. Gene targeting events will put the selectable marker under control of the promoter for the target gene. Gene targeting events are identified by selection for expression of the selectable marker. Another common strategy utilizes a positive-negative selection scheme. This scheme utilizes two selectable markers, one that confers resistance (R+) coupled with one that confers a sensitivity (S+), each with a promoter. When this polynucleotide is randomly inserted, the resulting phenotype is R+/S+. When a gene targeting event is generated, the two markers are uncoupled and the resulting phenotype is R+/S-. Examples of using positive-negative selection are found in Thykjær *et al.* (1997) *Plant Mol. Biol.* 35:523-530; and WO 01/66717, which are herein incorporated by reference.

Examples

The following are non-limiting examples intended to illustrate the invention. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Example 1

The example describes the identification of a microRNA

The following experiments were carried out on the *Arabidopsis thaliana* Col-0 ecotype. Plants were grown in long days (16 h light, 8 h dark) under cool white light at 22°C.

Arabidopsis plants were transformed by a modified version of the floral dip method, in which Agrobacterium cell suspension was applied to plants by direct watering from above. The T-DNA vector used, pHSbarENDs, contained four copies of the CAMV 35S enhancer adjacent to the right border, an arrangement similar to that described by Weigel *et al.* (*Plant Physiol.* 122:1003-1013, 2000). Transformed plants were selected with glufosinate (BASTA) and screened for flowering time, which resulted in the identification of the early-flowering EAT-D mutant. A single T-DNA cosegregating with early flowering was identified in EAT-D, and TAIL-PCR was performed to amplify sequences adjacent to the left and right borders of the T-DNA. To identify transcripts upregulated in the EAT-D mutant, we probed Northern blots containing RNA extracted from wild type (Col-0) and EAT-D plants. Probes for the genes At5g04270 and At5g04280 (GenBank NC_003076) did not detect any difference between wild type and EAT-D, whereas a probe from the intergenic region identified an ~1.4 kb transcript that was expressed at significantly higher levels in EAT-D than in wild type.

To isolate the full-length EAT cDNA, we performed 5'- and 3'-RACE-PCR with a GeneRacer kit (Invitrogen) that selects for 5'-capped mRNAs. Reverse transcription was carried out using an oligo-dT primer, and PCR utilized a gene-specific primer (SEQ ID NO: 45 5'-CTGTGCTCACGATCTTGTTGTTCTTGATC-3') paired with the 5' kit primer, or a second gene-specific primer (SEQ ID NO: 46 5'-GTCGGCGGATCCATGGAAGAAAGCTCATC-5') paired with the 3' kit primer.

The *Arabidopsis* EAT-D (Early Activation Tagged - Dominant) mutant was identified in an activation tagging population (Weigel *et al.*, *Plant Physiol.* 122:1003-1013, 2000). As evidenced by visual inspection and by measuring rosette leaf number (Table 1), the EAT-D mutant flowers extremely early. In addition, EAT-D displays floral defects that are virtually identical to those observed for strong apetala2 (ap2) mutant alleles (Bowman *et al.*, *Development* 112:1-20, 1991), including the complete absence of petals and the transformation of sepals to carpels. This ap2-like phenotype is only observed in EAT-D homozygotes, whereas both EAT-D heterozygotes and homozygotes are early flowering, indicating that the flowering time phenotype is more sensitive to EAT-D dosage than the ap2-like floral phenotype.

Table 1: Rosette leaf numbers for *Arabidopsis* lines

Genotype	rosette leaf no.	floral phenotype
Col-0	11.4 +/- 1.2	wild type
EAT-D	3.1 +/- 0.8	ap2
EAT-OX	2.0 +/- 0.2	ap2 + additional
eatdel	11.1 +/- 1.1	wild type
miR172a1-OX	2.1 +/- 0.3	ap2 + additional
LAT-D	22.5 +/- 2.1	wild type
At2g28550-OX	28.6 +/- 3.6	wild type
5-60120	10.2 +/- 1.4	wild type
2-28550	8.7 +/- 0.6	wild type
5-60120; 2-28550	6.0 +/- 0.8	wild type

We mapped the activation-tagged T-DNA insert in EAT-D to chromosome 5, in between the annotated genes At5g04270 and At5g04280. We then used 5'- and 3'-RACE PCR with primers located within this region to identify a 1.4 kb transcript (SEQ ID NO: 1), which we named EAT, that is upregulated in EAT-D. When the 1.4 kb EAT cDNA was fused to the constitutive CAMV 35S promoter and the resultant 35S::EAT construct was introduced into wild type (Col-0) plants by Agrobacterium-mediated transformation (Clough and Bent, *Plant J.* 16:735-743 1998), the 35S::EAT transformants displayed the identical early-flowering and ap2-like phenotypes seen for EAT-D (Table 1). Many of the 35S::EAT transformants occasionally displayed additional defects, including stigmatic papillae on cauline leaf margins and the formation of a complete or partial flower rather than a secondary inflorescence in the axils of cauline leaves. Ectopic

expression of the EAT gene in 35S::EAT plants, therefore, affects both flowering time and the specification of floral organ identity.

The EAT gene produces a 1417-nucleotide noncoding RNA that is predicted to be 5'-capped and polyadenylated, based on our RACE-PCR methodology. BLASTN and BLASTX searches of several databases with the EAT cDNA did not reveal extensive nucleotide or predicted amino acid sequence identity between EAT and any other gene. We did, however, identify a 21-nucleotide (nt) (SEQ ID NO: 4) stretch in the middle of the EAT transcript that is identical to miR172a-2, a recently identified miRNA (Park *et al.*, *Curr. Biol.* 12:1484-1495, 2002). To confirm the functional importance of the miR172a-2 sequence within the EAT cDNA, we generated a mutant form of EAT in which the miR172a-2 sequence was deleted, and made a construct consisting of this mutant EAT cDNA, eatdel, driven by the 35S promoter. Transgenic plants carrying this 35S::eatdel construct flowered with the same number of leaves as wild-type and had normal flowers (Table 1), indicating that the miR172a-2 sequence is necessary to confer both the flowering time and floral organ identity phenotypes seen in EAT-overexpressing lines.

As noted by Park *et al.* (*Curr. Biol.* 12:1484-1495, 2002), the 21-nt miR172a-2 miRNA has the potential to form an RNA duplex with a sequence near the 3' end of the coding region of AP2 (Table 2).

Table 2. Putative 21-nt miR172a-2/AP2 RNA duplex

Sequence	Duplex	SEQ ID NO:
AP2 RNA	5′ –**C**UGCAGCAUCAUCA**G**GAUUCU–3′	47
EAT miRNA	3′ –**U**ACGUCGUAGUAGU**U**CUAAGA–5′	48

The GU wobble in the duplex is underlined.

This particular region of the AP2 gene is poorly conserved at the nucleotide level among the AP2 family; nevertheless, the AP2 sequence (SEQ ID NO: 49) that is complementary to miR172a-2 is found in a similar location in three other *Arabidopsis* AP2 family members, At5g60120 (SEQ ID NO: 50), At2g28550 (SEQ ID NO: 51), At5g67180 (SEQ ID NO: 52). In addition, the sequence can be found at the corresponding positions of the maize AP2 genes indeterminate spikelet1 (Chuck *et al.*, *Genes. Dev.* 12:1145-1154 1998) (IDS1 (SEQ ID NO: 53)) and

glossy15 (Moose and Sisco, *Genes. Dev.* 10:3018-3027 1996) (GL15 (SEQ ID NO: 54)), and in AP2 family members from many other plant species, including soybean, rice, wheat, tomato and pea (not shown). The alignment of three *Arabidopsis* and two maize AP2 family members is shown in Table 3 below.

Table 3. Alignment of AP2 21-nt region (black bar) and surrounding sequence

AP2	ACCAAGTGTTGACAAATGCTGCAGCATCATCAGGATTCTCTCCTCATCATCACAATCAG
At5g60120	CACCGCCACTGTTTTCAAATGCAGCATCATCAGGATTCTCACTCTCAGCTACACGCCCT
At2g28550	CACCATTGTTCTCAGTTGCAGCAGCATCATCAGGATTCTCACATTTCCGGCCACAACCT
At5g67180	GAAATCGAGTGGTGGGAATGGCAGCATCATCAGGATTCTCTCCTCAACCTTCCCCTTAC
IDS1	ACGTGCCGTTGCACCACTCTGCAGCATCATCAGGATTCTCTACCGCCGCCGGGGCCAAC
GL15	ACGCCAGCAGCGCCGCCGCTGCAGCATCATCAGGATTCCCACTGTGGCAGCTGGGTGCG

There is an additional copy of the miR172a-2 miRNA in the *Arabidopsis* genome on chromosome 2 (miR172a-1, Fig. 2d), and miR172a-2 is highly similar to three other *Arabidopsis* loci. Like the miR172a-2 miRNA, all four reiterations of the sequence are in intergenic regions, *i.e.* in between the *Arabidopsis* genes currently annotated in GenBank. In addition, the sequence is found in ESTs from tomato, potato and soybean, and four copies were found in the genomic sequence of rice.

Example 2

This example describes the construction of expression vectors

To overexpress the EAT gene, we designed primers containing XhoI sites (SEQ ID NO: 55 5'- GACTACTCGAGCACCTCTCACTCCCTTTCTCTAAC-3' and SEQ ID NO: 56 5'- GACTACTCGAGGTTCTCAAGTTGAGCACTTGAAAAC-3') to amplify the entire EAT gene from Col-0 DNA. The PCR product was digested with XhoI and inserted into a modified pBluescriptSK+ vector (Stratagene, La Jolla, CA) that lacked BamHI and HindIII sites, to generate EATX4 (SEQ ID NO: 44). To generate the 35S::EAT transformants, the XhoI-cut EAT gene was inserted into the binary vector pBE851 in between a CAMV 35S promoter and b-phaseolin terminator, and Col-0 was transformed by floral dip. To generate the eatdel construct, two oligonucleotides were synthesized (SEQ ID NO: 57 5' GATCCATGGAAGAAAGCTCATCTGTCGTTGTTTGTAGGCGCAGCACCATTAA GATTCACATGGAAATTGATAAATAC-3' and SEQ ID NO: 58 5'- CCTAAATTAGGGTTTTGATATGTATATTCAACAATCGACGGCTACAAATACCTA

A-3') that completely recreated the BamHI/HindIII fragment of the EAT cDNA except that it lacked the 21 nt miR172a-2 sequence located within the fragment. These two oligos were annealed to their synthesized complementary strands (SEQ ID NO: 59 5'-
TAGGGTATTTATCAATTTCCATGTGAATCTTAATGGTGCTGCGCCTACAAACA
ACGACAGATGAGCTTTCTTCCATG-3' and SEQ ID NO: 60 5'-
AGCTTTAGGTATTTGTAGCCGTCGATTGTTGAATATACATATCAAAACCCTAAT
T-3') and ligated to EATX4 that had been digested with BamHI and HindIII, in a trimolecular ligation reaction. This resulted in the replacement of 159 bp of wild-type EAT sequence with the 138 bp mutant sequence. The eatdel cDNA was then subcloned into pBE851 and transformed as described above. BASTA was used to select in plants for both the EAT and eatdel overexpression constructs.

To test whether another member of the miR172 family, miR172a-1, would confer a phenotype similar to that of miR172a-2, we generated a construct containing the 35S promoter fused to the genomic region surrounding miR172a-1. Plants containing the 35S::miR172a-1 construct flowered early and displayed an ap2 phenotype (Table 1), indicating that miR172a-1 behaves in an identical manner to miR172a-2 when overexpressed.

All of the miR172 miRNA family members are located within a sequence context that allows an RNA hairpin to form (Figure 1). Presumably this hairpin is the substrate which is subsequently cleaved by a plant Dicer homolog to generate the mature miRNA. The location of the miRNA within the hairpin, *i.e.* on the 3' side of the stem, is conserved amongst all the members of the miR172 family, and this may reflect a structural requirement for processing of this particular miRNA family. The 21-nt miR172a-2 miRNA, therefore, is predicted to be a member of a family of miRNAs that have the capacity to regulate a subset of AP2 genes by forming an RNA duplex with a 21-nt cognate sequence in these genes.

Example 3:

The example describes the analysis of microRNA expression and AP2 expression

Total RNA was isolated from wild type and EAT-D whole plants that had already flowered, using TRIZOL reagent (Sigma). 50 mg of each RNA was subjected to electrophoresis on a 15% TBE-Urea Criterion gel (BioRad), electroblotted onto Hybond-N+ filter paper (Amersham) using a TransBlot-SD

apparatus (BioRad). The filter was then hybridized at 37°C overnight in UltraHyb-Oligo buffer (Ambion) with 32P-labeled oligos. The oligos were 30-mers that corresponded to either the sense or antisense strands of the miR172a-2 miRNA, with 4-5 nt of flanking sequence on each side. The filter was washed twice at 37°C, in buffer containing 2X SSC and 0.5% SDS. For S1 analysis, probe was made by end-labeling an oligo (SEQ ID NO: 61) (5'-ATGCAGCATCATCAAGATTCTCATATACAT-3') with T4 polynucleotide kinase and 32P. Hybridization and processing of S1 reactions were carried out using standard protocols. For developmental analysis of miR172a-2 and miR172a-1, total RNA was isolated from plants at the various stages and tissues indicated in Example 4, using an Rneasy kit (Qiagen). RT-PCR was carried out using standard protocols, and utilized oligos specific for sequences adjacent to miR172a-2 (SEQ ID NO: 62) (5'-GTCGGCGGATCCATGGAAGAAAGCTCATC-3' and (SEQ ID NO: 63) 5'-CAAAGATCGATCCAGACTTCAATCAATATC-3') or sequences adjacent to miR172a-1 (SEQ ID NO: 64) (5'-TAATTTCCGGAGCCACGGTCGTTGTTG-3' and (SEQ ID NO: 65) 5'-AATAGTCGTTGATTGCCGATGCAGCATC-3'). Oligos used to amplify the ACT11 (Actin) transcript were: (SEQ ID NO: 66) 5'-ATGGCAGATGGTGAAGACATTCAG-3', and (SEQ ID NO: 67) 5'-GAAGCACTTCCTGTGGACTATTGATG-3'. RT-PCR analysis of AP2 was performed on RNA from floral buds, and utilized the following oligos: (SEQ ID NO: 68) 5'-TTTCCGGGCAGCAGCAACATTGGTAG-3', and (SEQ ID NO: 69) 5'-GTTCGCCTAAGTTAACAAGAGGATTTAGG-3'. Oligos used to amplify the ANT transcript were: (SEQ ID NO: 70) 5'-GATCAACTTCAATGACTAACTCTGGTTTTC-3', and (SEQ ID NO: 71) 5'-GTTATAGAGAGATTCATTCTGTTTCACATG-3'.

Immunoblot analysis of AP2 was performed on proteins extracted from floral buds. Following electrophoresis on a 10% SDS-PAGE gel, proteins were transferred to a Hybond-P membrane (Amersham) and incubated with an antibody specific for AP2 protein (aA-20, Santa Cruz Biotechnology). The blot was processed using an ECL-plus kit (Amersham).

Northern analysis using probes both sense and antisense to the miR172a-2 miRNA identified a small single-stranded RNA of 21-25 nucleotides accumulating to much higher levels in EAT-D mutant plants relative to wild type. The small amount of transcript seen in wild type presumably represents endogenous levels

of not only the miR172a-2 miRNA but also its family members, which are similar enough to cross-hybridize with the probe. The predicted miR172a-2 hairpin is 117 nt in length (Fig. 1), a small amount of an ~100 nt transcript accumulating is detected in EAT-D, this likely represents partially processed miR172a-2 hairpin precursor. S1 nuclease mapping of the miR172a-2 miRNA provides independent confirmation of the 5' end of miR172a-2 reported by Park *et al.* (*Curr. Biol.* 12:1484-1495, 2002).

Example 4

The example describes the developmental pattern of EAT miRNA expression.

To address the wild-type expression pattern of miR172a-2 separate from its other *Arabidopsis* family members, RT-PCR was used to specifically detect a fragment of the 1.4 kb EAT full-length precursor transcript containing miR172a-2. EAT precursor transcript expression is temporally regulated, with little or no transcript detected two days after germination, and progressively more steady-state transcript accumulation seen as the plant approaches flowering. The precursor transcript of miR172a-1 showed a similar temporal pattern of expression. Both miR172a-2 and miR172a-1 precursor transcripts continue to be expressed after flowering has occurred, and accumulate in both leaves and floral buds. We were unable to detect expression of the precursors for the other miR172 family members, perhaps due to their exclusive expression in tissue types not included in this analysis, or because their precursor transcripts are too transient to detect. The temporal expression pattern seen for miR172a-2 and miR172a-1 is reminiscent of that observed for let-7 and lin-4, two miRNAs that control developmental timing in *C. elegans* (Feinbaum and Ambros, *Dev. Biol.* 210:87-95 1999; Reinhart *et al.*, *Nature* 403:901-906 2000).

Example 5

We assessed the levels of miR172 in various flowering time mutants, in an attempt to position miR172 within the known flowering time pathways. The levels of miR172 were not altered in any of the mutants tested, and the levels of the EAT transcript were identical in plants grown in long days versus plants grown in short days.

Example 6

The example describes evaluation of protein expression

Immunoblot analysis indicates that AP2 protein is reduced 3.5-fold in the EAT-D mutant relative to wild type, whereas the AP2 transcript is unaffected. This data suggests that the miR172a-2 miRNA negatively regulates AP2 by translational inhibition. The predicted near-perfect complementarity between the miR172a-2 miRNA and the AP2 target site would be predicted to trigger AP2 mRNA cleavage by the RNA interference (RNAi) pathway (Llave *et al.*, *Plant Cell* 14:1605-1619 2002; Hutvagner and Zamore, *Science* 297:2056-2060 2002). Indeed, others have proposed that many plant miRNAs enter the RNAi pathway exclusively due to their near-perfect complementarity to putative targets (Rhoades *et al.*, *Cell* 110:513-520 2002). While there is no evidence regarding the GU wobble base pair in the predicted miR172a-2/AP2 RNA duplex, it is conserved in all predicted duplexes between miR172 family members and their AP2 targets. Regardless of the mechanism, it is apparent from the AP2 expression data and the observed phenotype of EAT-D that AP2 is a target of negative regulation by miR172a-2, at least when miR172a-2 is overexpressed.

Example 7

In the same genetic screen that identified the early-flowering EAT-D mutant, we identified an activation-tagged late-flowering mutant, called LAT-D. The LAT-D mutant displays no additional phenotypes besides late flowering (Table 1), and the late-flowering phenotype cosegregated with a single T-DNA insertion. Sequence analysis of the T-DNA insert in LAT-D indicated that the 4X 35S enhancer was located approximately 5 kb upstream of At2g28550, which is one of the AP2-like target genes that are potentially regulated by miR172. RT-PCR analysis using primers specific for At2g28550 indicates that the transcript corresponding to this gene is indeed expressed at higher levels in the LAT-D mutant relative to wild type. To confirm that overexpression of At2g28550 causes late flowering, we fused a genomic region containing the entire At2g28550 coding region (from start to stop codon) to the 35S promoter, and created transgenic plants containing this construct. Transgenic 35S::At2g28550 plants flowered later than wild type plants, and were slightly later than the LAT-D mutant (Table 1).

This late flowering phenotype was observed in multiple independent transformants.

The fact that overexpression of At2g28550 causes late flowering suggests that miR172 promotes flowering in part by downregulating At2g28550. However, because miR172 appears to affect protein rather than transcript accumulation of its target genes, and because we do not have an antibody to the At2g28550 gene product, we decided to test this regulation indirectly via a genetic cross. A plant heterozygous for LAT-D was crossed to a plant homozygous for EAT-D, such that all F1 progeny would contain one copy of EAT-D and 50% of the F1 progeny would also have one copy of LAT-D. F1 progeny were scored for the presence or absence of the LAT-D allele by PCR, and also were scored for flowering time. All of the F1 plants were early flowering, regardless of whether or not they contained a copy of the LAT-D allele, indicating that EAT-D is epistatic to LAT-D. This result is consistent with the idea that miR172a-2, which is overexpressed in EAT-D, directly downregulates At2g28550, which is overexpressed in LAT-D.

Example 8

To assess the effects of reducing At2g28550 function, we identified plants containing a T-DNA insertion in the At2g28550 gene. In addition, we identified a T-DNA mutant for At2g60120, a closely related AP2-like gene that also contains the miR172 target sequence. Plants homozygous for either the At2g28550 insert or the At5g60120 insert were slightly early flowering relative to wild type (Table 1). The two mutants were crossed, and the double mutant was isolated by PCR genotyping. The At2g28550/At5g60120 double mutant was earlier flowering than either individual mutant (Table 1), suggesting that the genes have overlapping function. The early flowering phenotype of the At2g28550/At5g60120 double mutant is consistent with the idea that the early flowering phenotype of miR172-overexpressing lines is due to downregulation of several AP2-like genes, including At2g28550 and At5g60120. Interestingly, the At2g28550/At5g60120 double mutant is not as early as miR172-overexpressing lines (c.f. EAT-OX, Table 1), which suggests that other AP2-like targets of miR172, for example AP2 itself or At5g67180, also contribute to flowering time control. Because ap2 mutants are not early flowering, any potential negative regulation of flowering by AP2 must be normally masked by genetic redundancy.

Example 9

This example describes a method of target selection and method to design DNA constructs to generate miRNAs using the constructs of SEQ ID NOS: 3 and 44. Any sequence of interest can be selected for silencing by miRNA generated using the following method:

1. Choose a region from the coding strand in a gene of interest to be the target sequence. Typically, choose a region of about 10 – 50 nucleotides found in a similar location to the region targeted by EAT in AP2-like genes, which are regions about 100 nt upstream of the stop codon. The exact location of the target, however, does not appear to be critical. It is recommended to choose a region that has ~50% GC and is of high sequence complexity, *i.e.* no repeats or long polynucleotide tracts. It is also recommended that the chosen region ends with a T or A, such that the complementary miRNA will start with an A or U. This is to help ensure a lower stability at the 5' end of the miRNA in its double-stranded Dicer product form (Schwartz, et al. 2003 Cell 115:199-208). For example, in the miR172a-2 precursor, the miRNA sequence starts with an A, and many other miRNAs start with a U.

2. To use the construct of SEQ ID NO: 3, create a 21 nucleotide sequence complementary to the 21 nt target region (miRNA). Optionally, change a C in the miRNA to a T, which will generate a GU wobble with the target sequence, which mimics the GU wobble seen in EAT.

3. Create the 21 nucleotide "backside" sequence of the hairpin. This will be substantially complementary to the miRNA from step 2. Note, this backside sequence will also be substantially identical to the target sequence. Typically, introduce a few mismatches to make some bulges in the stem of the hairpin that are similar to the bulges in the original EAT hairpin. Optionally, introduce an A at the 3' end of the backside, to create mismatch at the 5' end of the miRNA. This last step may help ensure lower stability at the 5' end of the miRNA in its double-stranded Dicer product form (Schwartz, et al. 2003 Cell 115:199-208).

4. Replace the 21 nucleotide miRNA sequence and the 21 nucleotide "backside" sequence in the EAT BamHI/HindIII DNA construct (SEQ ID NO: 3) with the new miRNA and "backside" sequences from steps 2 and 3.

5. Use MFOLD (GCG, Accelrys, San Diego, CA), or an equivalent program, to compare the new hairpin from Step 4 with the original hairpin.

Generally, the sequence substantially replicate the structure of the original hairpin (Figure 1). It is predicted that the introduced bulges need not be exactly identical in length, sequence or position to the original. Examine the miRNA sequence in the hairpin for the relative stability of the 5' and 3' ends of the predicted dsRNA product of Dicer.

6. Generate four synthetic oligonucleotides of 76-77 nucleotides in length to produce two double-stranded fragments which comprise the BamHI and HindIII restriction sites, and a 4 nucleotide overhang to facilitate directional ligation which will recreate the BamHI/HindIII fragment. Design of the overhang can be done by one of skill in the art, the current example uses the 4 nucleotide region of positions 79-82 (CCTA) of SEQ ID NO: 3. Hence, for example:

Oligo 1 will have an unpaired BamHI site at the 5' end, and will end with the nucleotide at position 78 of SEQ ID NO: 3.

Oligo 2 will have the nucleotides of position 79-82 (CCTA) unpaired at the 5' end, and will terminate just before the HindIII site (or positions 151-154 in SEQ ID NO: 3).

Oligo 3 will be essentially complementary to Oligo 1, (nucleotides 5-78 of SEQ ID NO: 3), and will terminate with 4 nucleotides complementary to nucleotides 1-4 (CCTA) of Oligo 2.

Oligo 4 will be essentially complementary to Oligo 2 beginning at the nucleotide of position 5, and will terminate with the HindIII site at the 3' end.

Anneal the oligonucleotides to generate two fragments to be used in a subsequence ligation reaction with the plasmid sequence.

Optionally, two synthetic oligonucleotides comprising attB sequences can be synthesized and annealed to create an attB-flanked miRNA precursor that is then integrated into a vector using recombinational cloning (GATEWAY, InVitrogen Corp., Carlsbad, CA).

7. Ligate the two DNA fragments from Step 6 in a trimolecular ligation reaction with a plasmid cut with BamHI/HindIII. The current example uses the modified pBluescript SK+ plasmid of SEQ ID NO: 44, which comprises the 1.4kb EAT sequence of SEQ ID NO: 1, digested with BamHI/HindIII and gel purified away from the small fragment using standard molecular biological techniques. The new designed miRNA to the gene of interest has replaced the previous miRNA.

If an attB-flanked sequence is used from Step 6, the BP and LR recombination reactions (GATEWAY, InVitrogen Corp., Carlsbad, CA) can be used to insert the modified hairpin into a destination vector comprising the full-length miR172a-2 precursor.

8. The plasmid from Step 7, subject to any other preparations or modifications as needed, is used to transform the target organism using techniques appropriate for the target.

9. Silencing of the target gene can be assessed using techniques well-known in the art, for example, Northern blot analysis, immunoblot analysis if the target gene of interest encodes a polypeptide, and any phenotypic screens relevant to the target gene, for example flowering time, or floral morphology.

Example 10

Described in this example are methods one may use for introduction of a polynucleotide or polypeptide into a plant cell.

A. Maize particle-mediated DNA delivery

A DNA construct can be introduced into maize cells capable of growth on suitable maize culture medium. Such competent cells can be from maize suspension culture, callus culture on solid medium, freshly isolated immature embryos or meristem cells. Immature embryos of the Hi-II genotype can be used as the target cells. Ears are harvested at approximately 10 days post-pollination, and 1.2-1.5mm immature embryos are isolated from the kernels, and placed scutellum-side down on maize culture medium.

The immature embryos are bombarded from 18-72 hours after being harvested from the ear. Between 6 and 18 hours prior to bombardment, the immature embryos are placed on medium with additional osmoticum (MS basal medium, Musashige and Skoog, 1962, *Physiol. Plant* 15:473-497, with 0.25 M sorbitol). The embryos on the high-osmotic medium are used as the bombardment target, and are left on this medium for an additional 18 hours after bombardment.

For particle bombardment, plasmid DNA (described above) is precipitated onto 1.8 mm tungsten particles using standard CaCl2- spermidine chemistry (see, for example, Klein *et al.*, 1987, *Nature* 327:70-73). Each plate is bombarded once at 600 PSI, using a DuPont Helium Gun (Lowe *et al.*, 1995, *Bio/Technol* 13:677-

682). For typical media formulations used for maize immature embryo isolation, callus initiation, callus proliferation and regeneration of plants, see Armstrong, C., 1994, In "The Maize Handbook", M. Freeling and V. Walbot, eds. Springer Verlag, NY, pp 663-671.

Within 1-7 days after particle bombardment, the embryos are moved onto N6-based culture medium containing 3 mg/l of the selective agent bialaphos. Embryos, and later callus, are transferred to fresh selection plates every 2 weeks. The calli developing from the immature embryos are screened for the desired phenotype. After 6-8 weeks, transformed calli are recovered.

B. Soybean transformation

Soybean embryogenic suspension cultures are maintained in 35 ml liquid media SB196 or SB172 in 250 ml Erlenmeyer flasks on a rotary shaker, 150 rpm, 26C with cool white fluorescent lights on 16:8 hr day/night photoperiod at light intensity of 30-35 uE/m2s. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of fresh liquid media. Alternatively, cultures are initiated and maintained in 6-well Costar plates.

SB 172 media is prepared as follows: (per liter), 1 bottle Murashige and Skoog Medium (Duchefa # M 0240), 1 ml B5 vitamins 1000X stock, 1 ml 2,4-D stock (Gibco 11215-019), 60 g sucrose, 2 g MES, 0.667 g L-Asparagine anhydrous (GibcoBRL 11013-026), pH 5.7. SB 196 media is prepared as follows: (per liter) 10ml MS FeEDTA, 10ml MS Sulfate, 10ml FN-Lite Halides, 10ml FN-Lite P,B,Mo, 1ml B5 vitamins 1000X stock, 1 ml 2,4-D, (Gibco 11215-019), 2.83g KNO3 , 0.463g (NH4)2SO4, 2g MES, 1g Asparagine Anhydrous, Powder (Gibco 11013-026), 10g Sucrose, pH 5.8. 2,4-D stock concentration 10 mg/ml is prepared as follows: 2,4-D is solubilized in 0.1 N NaOH, filter-sterilized, and stored at -20°C. B5 vitamins 1000X stock is prepared as follows: (per 100 ml) - store aliquots at -20°C, 10 g myo-inositol, 100 mg nicotinic acid, 100 mg pyridoxine HCl, 1 g thiamin.

Soybean embryogenic suspension cultures are transformed with various plasmids by the method of particle gun bombardment (Klein *et al.*, 1987 *Nature* 327:70. To prepare tissue for bombardment, approximately two flasks of suspension culture tissue that has had approximately 1 to 2 weeks to recover since its most recent subculture is placed in a sterile 60 x 20 mm petri dish

containing 1 sterile filter paper in the bottom to help absorb moisture. Tissue (*i.e.* suspension clusters approximately 3-5 mm in size) is spread evenly across each petri plate. Residual liquid is removed from the tissue with a pipette, or allowed to evaporate to remove excess moisture prior to bombardment. Per experiment, 4 - 6 plates of tissue are bombarded. Each plate is made from two flasks.

To prepare gold particles for bombardment, 30 mg gold is washed in ethanol, centrifuged and resuspended in 0.5 ml of sterile water. For each plasmid combination (treatments) to be used for bombardment, a separate micro-centrifuge tube is prepared, starting with 50 µl of the gold particles prepared above. Into each tube, the following are also added; 5µl of plasmid DNA (at 1µg/µl), 50µl CaCl2, and 20µl 0.1 M spermidine. This mixture is agitated on a vortex shaker for 3 minutes, and then centrifuged using a microcentrifuge set at 14,000 RPM for 10 seconds. The supernatant is decanted and the gold particles with attached, precipitated DNA are washed twice with 400 µl aliquots of ethanol (with a brief centrifugation as above between each washing). The final volume of 100% ethanol per each tube is adjusted to 40µl, and this particle/DNA suspension is kept on ice until being used for bombardment.

Immediately before applying the particle/DNA suspension, the tube is briefly dipped into a sonicator bath to disperse the particles, and then 5 µL of DNA prep is pipetted onto each flying disk and allowed to dry. The flying disk is then placed into the DuPont Biolistics PDS1000/HE. Using the DuPont Biolistic PDS1000/HE instrument for particle-mediated DNA delivery into soybean suspension clusters, the following settings are used. The membrane rupture pressure is 1100 psi. The chamber is evacuated to a vacuum of 27-28 inches of mercury. The tissue is placed approximately 3.5 inches from the retaining/stopping screen (3rd shelf from the bottom). Each plate is bombarded twice, and the tissue clusters are rearranged using a sterile spatula between shots.

Following bombardment, the tissue is re-suspended in liquid culture medium, each plate being divided between 2 flasks with fresh SB196 or SB172 media and cultured as described above. Four to seven days post-bombardment, the medium is replaced with fresh medium containing a selection agent. The selection media is refreshed weekly for 4 weeks and once again at 6 weeks. Weekly replacement after 4 weeks may be necessary if cell density and media turbidity is high.

Four to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated, green tissue is removed and inoculated into 6-well microtiter plates with liquid medium to generate clonally-propagated, transformed embryogenic suspension cultures.

Each embryogenic cluster is placed into one well of a Costar 6-well plate with 5mls fresh SB196 media with selection agent. Cultures are maintained for 2-6 weeks with fresh media changes every 2 weeks. When enough tissue is available, a portion of surviving transformed clones are subcultured to a second 6-well plate as a back-up to protect against contamination.

To promote in vitro maturation, transformed embryogenic clusters are removed from liquid SB196 and placed on solid agar media, SB 166, for 2 weeks. Tissue clumps of 2 - 4 mm size are plated at a tissue density of 10 to 15 clusters per plate. Plates are incubated in diffuse, low light (< 10 µE) at 26 +/- 1°C. After two weeks, clusters are subcultured to SB 103 media for 3 - 4 weeks.

SB 166 is prepared as follows: (per liter), 1 pkg. MS salts (Gibco/ BRL - Cat# 11117-017), 1 ml B5 vitamins 1000X stock, 60 g maltose, 750 mg MgCl2 hexahydrate, 5 g activated charcoal, pH 5.7, 2 g gelrite. SB 103 media is prepared as follows: (per liter), 1 pkg. MS salts (Gibco/BRL - Cat# 11117-017), 1 ml B5 vitamins 1000X stock, 60 g maltose, 750 mg MgCl2 hexahydrate, pH 5.7, 2 g gelrite. After 5-6 week maturation, individual embryos are desiccated by placing embryos into a 100 X 15 petri dish with a 1cm2 portion of the SB103 media to create a chamber with enough humidity to promote partial desiccation, but not death.

Approximately 25 embryos are desiccated per plate. Plates are sealed with several layers of parafilm and again are placed in a lower light condition. The duration of the desiccation step is best determined empirically, and depends on size and quantity of embryos placed per plate. For example, small embryos or few embryos/plate require a shorter drying period, while large embryos or many embryos/plate require a longer drying period. It is best to check on the embryos after about 3 days, but proper desiccation will most likely take 5 to 7 days. Embryos will decrease in size during this process.

Desiccated embryos are planted in SB 71-1 or MSO medium where they are left to germinate under the same culture conditions described for the

suspension cultures. When the plantlets have two fully-expanded trifoliate leaves, germinated and rooted embryos are transferred to sterile soil and watered with MS fertilizer. Plants are grown to maturity for seed collection and analysis. Healthy, fertile transgenic plants are grown in the greenhouse.

SB 71-1 is prepared as follows: 1 bottle Gamborg's B5 salts w/ sucrose (Gibco/BRL - Cat# 21153-036), 10 g sucrose, 750 mg MgCl2 hexahydrate, pH 5.7, 2 g gelrite. MSO media is prepared as follows: 1 pkg Murashige and Skoog salts (Gibco 11117-066), 1 ml B5 vitamins 1000X stock, 30 g sucrose, pH 5.8, 2g Gelrite.

Example 11

This example describes the design and synthesis of miRNA targets and hairpins directed to various gene targets found in maize, soy, and/or *Arabidopsis*, using the method described in Example 9.

A. Targeting *Arabidopsis* AGAMOUS, At4g18960

The miRNA sequence of SEQ ID NO: 4 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 12-15, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

Arabidopsis thaliana Col-0 was transformed and grown as described in Example 1. After transformation with a vector comprising the miRNA of SEQ ID NO: 4, 88% of the transformants exhibited a mutant AGAMOUS (*ag*) floral phenotype, characterized by the conversion of stamens to petals in whorl 3, and carpels to another *ag* flower in whorl 4 (Bowman, *et al.* (1991) The Plant Cell 3:749-758). The mutant phenotype varied between transformants, with approximately 1/3 exhibiting a strong *ag* phenotype, 1/3 exhibiting an intermediate *ag* phenotype, and 1/3 exhibiting a weak *ag* phenotype. Gel electrophoresis and Northern Blot analysis of small RNAs isolated from the transformants demonstrated that the degree of the mutant ag phenotype was directly related to the level of antiAG miRNA, with the strongest phenotype having the highest accumulation of the processed miRNA (~ 21 nt).

B. Targeting Arabidopsis Apetela3 (AP3), At3g54340

Two miRNA targets from AP3 were selected and oligonucleotides designed.

The miRNA sequence of SEQ ID NO: 5 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 16-19, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

The miRNA sequence of SEQ ID NO: 6 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 20-23, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

Arabidopsis thaliana Col-0 was transformed and grown as described in Example 1. After transformation with a vector comprising the miRNA of SEQ ID NO: 5, the transformants had novel leaf and floral phenotypes, but did not exhibit any mutant AP3 phenotype. Gel electrophoresis and Northern analysis of RNA isolated from 2 week old rosette leaf tissue from the transformants demonstrated that the highest accumulation of the processed miRNA (~ 21 nt) corresponded to the "backside" strand of the precursor, which evidently silenced a different target sequence to produce the novel leaf and floral phenotypes.

A new target sequence was selected, with the correct asymmetry in order for the miRNA target strand to be selected during incorporation into RISC (Schwartz, et al. 2003 Cell 115:199-208). The miRNA sequence of SEQ ID NO: 6 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 20-23, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44. Greater than 90% of the transformants showed silencing for the AP3 gene, as demonstrated by floral phenotype and electrophoretic analysis. An approximately 21 nt miRNA (antiAP3b) was detected at high levels in the transgenic plants, and not in wild type control plants. RT-PCR analysis confirmed that the amount of AP3 transcript was reduced in the transformants, as compared to wild type control plants.

C. Targeting Maize Phytoene Desaturase

Two miRNA targets from phytoene desaturase (PDS) were selected and oligonucleotides designed.

The miRNA sequence of SEQ ID NO: 7 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 24-27, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

The miRNA sequence of SEQ ID NO: 8 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 28-31, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

D. Targeting Maize Phytic Acid biosynthetic enzymes

Three maize phytic acid biosynthetic enzyme gene targets were selected and miRNA and oligonucleotides designed. Inositol polyphosphate kinase-2 polynucleotides are disclosed in WO 02/059324, herein incorporated by reference. Inositol 1,3,4-trisphosphate 5/6-kinase polynucleotides are disclosed in WO 03/027243, herein incorporated by reference. Myo-inositol 1-phosphate synthase polynucleotides are disclosed in WO 99/05298, herein incorporated by reference.

Inositol polyphosphate kinase-2 (IPPK2)

The miRNA sequence of SEQ ID NO: 9 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 32-35, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

Inositol 1,3,4-trisphosphate 5/6-kinase-5 (ITPK5)

The miRNA sequence of SEQ ID NO: 10 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 36-39, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

Myo-inositol 1-phosphate synthase (mi1ps)

The miRNA sequence of SEQ ID NO: 11 was selected and designed. The sequence is put into the BamHI/HindIII hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 40-43, and ligating them into the BamHI/HindIII backbone fragment of SEQ ID NO: 44.

E. Targeting Soy Apetela2-like sequences (AP2)

The same EAT (miR172a-2) construct, comprising SEQ ID NO: 1, used for *Arabidopsis* transformation was used to transform soybean. This construct has a miRNA template sequence which encodes the miRNA of SEQ ID NO: 48. The construct was created using a PCR amplification of miR172a-2 precursor sequence from *Arabidopsis*, restriction digestion, and ligation as described in Example 2.

Soybean tissue was transformed and grown essentially as described in Example 10. After transformation, 42% of the transformants exhibited a mutant phenotype, characterized by the conversion of sepals to leaves. Plants exhibiting the strongest phenotypes were sterile, and produced no seed. Both the homeotic conversion of the organs and the effects on fertility are similar to that seen for *ap2* mutant alleles in *Arabidopsis*. Small RNA gel electrophoresis and Northern analysis, probed with an oligonucleotide probe antisense to miR172, showed accumulation of miR172 in the transgenic lines. A small amount of endogenous soy miR172 is also detected in the soy control line. The degree of the mutant phenotype was directly related to the level of miRNA, with the strongest phenotype having the highest accumulation of the processed miRNA (~ 21 nt).

F. Targeting *Arabidopsis* AP2-like genes

The miRNA sequence of SEQ ID NO: 72 was selected and designed. The sequence is put into the attB hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 73-74, and performing the BP recombination reaction (GATEWAY) to generate the attL intermediate. This intermediate is used in the LR reaction to recombine with the destination vector, generally described in Example 12, comprising the EAT full-length precursor containing attR sites, and negative selection markers in place of the hairpin. The product of this reaction comprises the miR172a-2 precursor hairpin cassette flanked by attR sites (*i.e.*, the hairpin replaces the marker cassette).

G. Targeting *Arabidopsis* Fatty Acid Desaturase (FAD2)

The miRNA sequence of SEQ ID NO: 75 was selected and designed based on the sequence of NM_112047 (At3g12120). The sequence is put into the attB hairpin

cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 76-77, and performing the BP recombination reaction (GATEWAY) to generate the attL intermediate. This intermediate is used in the LR reaction to recombine with the destination vector, generally described in Example 12, comprising the EAT full-length precursor containing attR sites, and negative selection markers in place of the hairpin. The product of this reaction comprises the FAD2 miRNA precursor hairpin cassette flanked by attR sites (*i.e.*, the hairpin replaces the marker cassette). The effect of the anti-FAD2 miRNA can be determined by fatty acid analysis to determine the change in the fatty acid profile, for example, see Wu, *et al.* (1997) *Plant Physiol.* 113:347-356, herein incorporated by reference.

H. Targeting Arabidopsis Phytoene Desaturase (PDS)

The miRNA sequence of SEQ ID NO: 78 was selected and designed based on the sequence of NM_202816 (At4g14210). The sequence is put into the attB hairpin cassette by annealing the synthetic oligonucleotides of SEQ ID NOS: 79-80, and performing the BP recombination reaction (GATEWAY) to generate the attL intermediate. This intermediate is used in the LR reaction to recombine with the destination vector, generally described in Example 12, comprising the EAT full-length precursor containing attR sites, and negative selection markers in place of the hairpin. The product of this reaction comprises the PDS miRNA precursor hairpin cassette flanked by attR sites (*i.e.*, the hairpin replaces the marker cassette). Transgenic plants containing the antiPDS construct were photobleached upon germination in greater than about 90% of the lines, indicating silencing of PDS.

Example 12

This example describes the construction of expression vectors using recombinational cloning technology.

The vector described in Example 2 (SEQ ID NO: 44) was modified to incorporate att recombination sites to facilitate recombinational cloning using GATEWAY technology (InVitrogen, Carlsbad, CA). The BamHI/HindIII segment was replaced with a sequence comprising in the following order: attR1 – CAM – ccdB – attR2. Upon recombination (BP + LR) with oligos containing attB sites

flanking the miRNA hairpin precursor construct, the selectable markers are replaced by the miRNA hairpin precursor.

Example 13

This example summarizes the target sequences and oligos used for miRNA silencing constructs as described in the examples.

Table 4:

Organism	**Target gene**	**miRNA name**	**miRNA template**	**Precursor oligos SEQ ID NOS**
Arabidopsis	AP2-like	miR172-a2	SEQ ID NO: 86	55-56 (PCR)
	none	EATdel	none	57-60
	AGAMOUS	antiAG	SEQ ID NO: 4	12-15
	APETELA3 (a)	antiAP3a	SEQ ID NO: 5	16-19
	APETELA3 (b)	antiAP3b	SEQ ID NO: 6	20-23
Corn	PDS1	antiPDS1	SEQ ID NO: 7	24-27
	PDS2	antiPDS1	SEQ ID NO: 8	28-31
	IPPK2	antiIPPK2	SEQ ID NO: 9	32-35
	ITPK5	antiITPK5	SEQ ID NO: 10	36-39
	MI1PS	antiMI1PS	SEQ ID NO: 11	40-43
Soybean	AP2-like	miR172a-2	SEQ ID NO: 86	55-56 (PCR)
Arabidopsis	AP2-like	miR172a-2	SEQ ID NO: 72	73-74
	FAD2	antiFAD2	SEQ ID NO: 75	76-77
	PDS	antiAtPDS	SEQ ID NO: 78	79-80
Corn	miR172b	miR172	SEQ ID NO: 92	91
	PDS	antiZmPDS	SEQ ID NO: 95	94

Example 14

This example describes the identification and isolation of genomic corn miR172 precursors.

The Genome Survey Sequence (GSS) database of the National Center for Biotechnology Information (NCBI) was searched using the 21nt miR172a-2 sequence in order to identify genomic corn sequences containing miR172 precursor sequence. Several corn miR172 precursors were identified, and named miR172a – miR172e (SEQ ID NOS: 81-85) as summarized in Table 5. Each sequence was imported into Vector NTI (InVitrogen, Carlsbad, CA) and contig analyses done. The analysis identified four distinct loci, each with a unique consensus sequence. A region of about 200 nucleotides surrounding the miRNA sequence from each locus was examined for secondary structure folding using RNA Structure software (Mathews, *et al.* 2004 *PNAS USA* 101:7287-7292, herein incorporated by reference). The results of this analysis identified the hairpin precursors of each of the corn sequences miR172a-e.

Oligonucleotides were designed in order amplify miR172a or miR172b from a B73 genomic corn library, these primers also add restriction enzyme recognition sites in order to facilitate cloning (BamHI or EcoRV). Alternatively, PCR primers designed to create att sites for recombinational cloning could be used. After PCR amplification, the products were isolated, purified, and the confirmed by sequence analysis. Once confirmed, these sequences were inserted into a construct comprising the corn ubiquitin (UBI) promoter. This construct can be used for further transformation vector construction, for example, with the addition of att sites, the GATEWAY system can be used.

The following PCR primers were used to amplify a sequence comprising the hairpin precursor of corn miR172a

Forward primer (SEQ ID NO: 87): 5' GGATCCTCTGCACTAGTGGGGTTATT 3'

Reverse primer (SEQ ID NO: 88): 5'GATATCTGCAACAGTTTACAGGCGTT 3'

The following PCR primers were used to amplify a sequence comprising the hairpin precursor of corn miR172b

Forward primer (SEQ ID NO: 89): 5' GGATCCCATGATATAGATGATGCTTG 3'

Reverse primer (SEQ ID NO: 90): 5' GATATCAAGAGCTGAGGACAAGTTTT 3'

Table 5. Corn miR172 precursors and positions of hairpin, & miRNA duplex components

Precursor	NCBI ID	Corn Line	SEQ ID NO:	Length	Hairpin	Backside	miRNA
miR172a	CG090465	B73	81	907	508-598	512-532	574-594
miR172b	BZ401521 and BZ4011525	B73 (both)	82	1128	551-654	567-587	620-640
miR172c	CG247934	B73	83	912	230-400	250-270	364-384
miR172d	CG097860 and BZ972414	B73	84	1063	351-520	361-381	466-486
miR172e	CG065885 and CC334589	B73 (both)	85	1738	913-1072	931-951	1033-1053

Example 15

This example describes the design and synthesis of miRNA targets and hairpins directed to various gene targets found in maize, for use with the corn miR172b miRNA precursor.

A. miR172b target in corn

Similar to the *Arabidopsis* EAT examples, the corn miR172b hairpin precursor will be tested by overexpression in corn. The precursor sequence comprising the miRNA template is shown in SEQ ID NO: 91. The miRNA is shown in SEQ ID NO: 92, and the backside of the miRNA duplex is shown in SEQ ID NO: 93. A double-stranded DNA molecule comprising the miRNA precursor and restriction enzyme overhangs, for BamHI and KpnI, is created by annealing the oligonucleotides of SEQ ID NOS: 97 and 98.

B. Phytoene Desaturase (PDS)

An oligonucleotide comprising the the miRNA template is shown in SEQ ID NO: 94. The miRNA directed to PDS is shown in SEQ ID NO: 92, and the backside of the miRNA duplex is shown in SEQ ID NO: 93. A double-stranded DNA molecule comprising the miRNA precursor and restriction enzyme overhangs, for BamHI and KpnI, is created by annealing the oligonucleotides of SEQ ID NOS: 99 and 100.

The oligonucleotides of this example can be inserted into vectors for transformation of corn using standard cloning techniques, including restriction digestion and ligation, and/or recombinational cloning such as GATEWAY.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, patent applications, and computer programs cited herein are hereby incorporated by reference.

WHAT IS CLAIMED

1. A method of inhibiting expression of a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide, the polynucleotide comprising in the following order:

(i) at least about 20 contiguous nucleotides in the region from nucleotides 1-38 of SEQ ID NO: 3,

(ii) a first oligonucleotide of 10 to about 50 contiguous nucleotides, wherein the first oligonucleotide is substantially complementary to a second oligonucleotide,

(iii) at least about 20 contiguous nucleotides in the region from nucleotides 60-106 of SEQ ID NO: 3,

(iv) the second oligonucleotide of about 10 to about 50 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and wherein the second oligonucleotide is substantially complementary to the target sequence, and

(v) at least about 20 contiguous nucleotides in the region from nucleotides 128-159 of SEQ ID NO:3;

wherein the polynucleotide encodes an RNA precursor capable of forming a hairpin, and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

2. The method of claim 1, wherein the nucleic acid construct further comprises a promoter operably linked to the polynucleotide

3. The method of claim 1, wherein the cell is a plant cell.

4. A cell comprising the nucleic acid construct of claim 1.

5. The cell of claim 4, wherein the cell is a plant cell.

6. The method of claim 1, wherein target sequence expression is inhibited by at least 10%.

7. The method of claim 1, wherein inhibition of the target sequence generates a loss-of-function phenotype.

8. The method of claim 2, wherein the promoter is a pathogen-inducible promoter and inhibition of the target sequence confers resistance to a pathogen.

9. An isolated polynucleotide comprising in the following order at least 20 contiguous nucleotides in the region from nucleotides 1-38 of SEQ ID NO: 3, a first oligonucleotide of about 10 to about 50 contiguous nucleotides, wherein the first oligonucleotide is substantially complementary to a second oligonucleotide, at least about 20 contiguous nucleotides in the region from nucleotides 60-106 of SEQ ID NO: 3, a second oligonucleotide of about 10 to about 50 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and the second oligonucleotide is substantially complementary to the target sequence, and at least about 20 contiguous nucleotides in the region from nucleotides 128-159 of SEQ ID NO: 3, wherein the polynucleotide encodes an RNA precursor capable of forming a hairpin.

10. The isolated polynucleotide of claim 9, further comprising an operably linked promoter.

11. A cell comprising the isolated polynucleotide of claim 9.

12. The cell of claim 11, wherein the cell is a plant cell.

13. A transgenic plant comprising the isolated polynucleotide of claim 9.

14. A transgenic seed comprising the isolated polynucleotide of claim 9.

15. The isolated polynucleotide of claim 10, wherein the promoter is a pathogen-inducible promoter.

16. The isolated polynucleotide of claim 9, wherein the polynucleotide suppresses expression of a target sequence.

17. A method of inhibiting expression of a target sequence in a cell comprising:

(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide, wherein the polynucleotide comprises in the following order:

(i) nucleotides 1-38 of SEQ ID NO: 3,

(ii) a first oligonucleotide of 21 contiguous nucleotides, wherein the first oligonucleotide is substantially complementary to a second oligonucleotide,

(iii) nucleotides 60-106 of SEQ ID NO: 3,

(iv) a second oligonucleotide of 21 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and wherein the second oligonucleotide is substantially complementary to the target sequence, and

(v) nucleotides 128-159 of SEQ ID NO:3;

wherein the polynucleotide encodes an RNA precursor capable of forming a hairpin, and

(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

18. An isolated polynucleotide comprising in the following order nucleotides 1-38 of SEQ ID NO: 3, a first oligonucleotide of 21 contiguous nucleotides, wherein the first oligonucleotide is substanitially complementary to a second oligonucleotide, nucleotides 60-106 of SEQ ID NO: 3, a second oligonucleotide of 21 contiguous nucleotides, wherein the second oligonucleotide encodes a miRNA, and the second oligonucleotide is substantially complementary to the target sequence, and nucleotides 128-159 of SEQ ID NO:3, wherein the polynucleotide encodes an RNA precursor capable of forming a hairpin.

19. A method of inhibiting expression of a target sequence in a cell comprising:
(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide encoding a modified plant miRNA precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide encodes a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin; and
(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

20. A method of inhibiting expression of a target sequence in a cell comprising:
(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide encoding a modified plant miR172 miRNA precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide encodes a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin; and
(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

21. The method of claim 20, wherein the plant miR172 miRNA precursor is from a dicot or a monocot.

22. The method of claim 20, wherein the plant miR172 miRNA precursor is from a plant selected from the group consisting of Arabidopsis, tomato, soybean, rice, and corn.

23. The method of claim 20, wherein the plant miR172 miRNA precursor comprises SEQ ID NO: 3.

24. A method of inhibiting expression of a target sequence in a cell comprising:
(a) introducing into the cell a nucleic acid construct comprising a promoter operably linked to a polynucleotide encoding a modified Arabidopsis miR172 miRNA precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide encodes a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin; and
(b) expressing the nucleic acid construct for a time sufficient to produce the miRNA, wherein the miRNA inhibits expression of the target sequence.

25. The method of claim 24, wherein the Arabidopsis miR172 miRNA precursor comprises SEQ ID NO: 3.

26. A cell produced by the method of claim 1.

27. The cell of claim 26, wherein the cell is a plant cell.

28. The cell of claim 27, wherein the plant cell is from a monocot or a dicot.

29. The cell of claim 28, wherein the cell is selected from the group consisting of corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, *Arabidopsis*, and tobacco.

30. The cell of claim 29, wherein the cell is from *Arabidopsis*.

31. The cell of claim 29, wherein the cell is from corn.

32. A cell produced by the method of claim 19.

33. The cell of claim 32, wherein the cell is a plant cell.

34. The cell of claim 33, wherein the plant cell is from a monocot or a dicot.

35. The cell of claim 34, wherein the cell is selected from the group consisting of corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, Arabidopsis, and tobacco.

36. The cell of claim 35, wherein the cell is from *Arabidopsis*.

37. The cell of claim 35, wherein the cell is from corn.

38. A cell produced by the method of claim 20.

39. The cell of claim 38, wherein the cell is a plant cell.

40. The cell of claim 39, wherein the plant cell is from a monocot or a dicot.

41. The cell of claim 40, wherein the cell is selected from the group consisting of corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, *Arabidopsis*, and tobacco.

42. The cell of claim 41, wherein the cell is from *Arabidopsis*.

43. The cell of claim 41, wherein the cell is from corn.

44. A cell produced by the method of claim 24.

45. The cell of claim 44, wherein the cell is a plant cell.

46. The cell of claim 45, wherein the plant cell is from a monocot or a dicot.

47. The cell of claim 46, wherein the cell is selected from the group consisting of corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, Arabidopsis, and tobacco.

48. The cell of claim 47, wherein the cell is from *Arabidopsis*.

49. The cell of claim 47, wherein the cell is from corn.

50. The method of claim 3, the method further comprising producing a transformed plant, wherein the plant comprises the nucleic acid construct which encodes the miRNA.

51. A plant produced by the method of claim 50.

52. The method of claim 19, wherein the cell is a plant cell, the method further comprising producing a transformed plant, wherein the plant comprises the nucleic acid construct which encodes the miRNA.

53. A plant produced by the method of claim 52.

54. The method of claim 20, wherein the cell is a plant cell, the method further comprising producing a transformed plant, wherein the plant comprises the nucleic acid construct which encodes the miRNA.

55. A plant produced by the method of claim 50.

56. The method of claim 24, wherein the cell is a plant cell, the method further comprising producing a transformed plant, wherein the plant comprises the nucleic acid construct which encodes the miRNA.

57. A plant produced by the method of claim 56.

58. The plant of claim 57, wherein the plant is a monocot or a dicot.

59. The plant of claim 58, wherein the plant is selected from the group consisting of corn, wheat, rice, barley, oats, sorghum, millet, sunflower, safflower, cotton, soy, canola, alfalfa, *Arabidopsis*, and tobacco.

60. The plant of claim 59, wherein the plant is *Arabidopsis*.

61. The plant of claim 59, wherein the plant is corn.

62. An isolated polynucleotide comprising a polynucleotide which encodes a modified plant miRNA precursor, the modified precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide comprises a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin.

63. A cell comprising the isolated polynucleotide of claim 62.

64. The cell of claim 63, wherein the cell is a plant cell.

65. An isolated polynucleotide comprising a polynucleotide encoding a modified plant miR172 miRNA precursor, the modified precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide comprises a miRNA substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin.

66. A cell comprising the isolated polynucleotide of claim 65.

67. The cell of claim 66, wherein the cell is a plant cell.

68. An isolated polynucleotide comprising polynucleotide encoding a modified Arabidopsis miR172 miRNA precursor, the modified precursor comprising a first and a second oligonucleotide, wherein at least one of the first or the

second oligonucleotides is heterologous to the precursor, wherein the first oligonucleotide is substantially complementary to the second oligonucleotide, and the second oligonucleotide comprises a miRNA which is substantially complementary to the target sequence, wherein the precursor is capable of forming a hairpin.

69. A cell comprising the isolated polynucleotide of claim 68..

70. The cell of claim 69, wherein the cell is a plant cell.

71. The cell of claim 70, wherein the plant cell is from a dicot or a monocot.

72. The cell of claim 71, wherein the cell is from *Arabidopsis*.

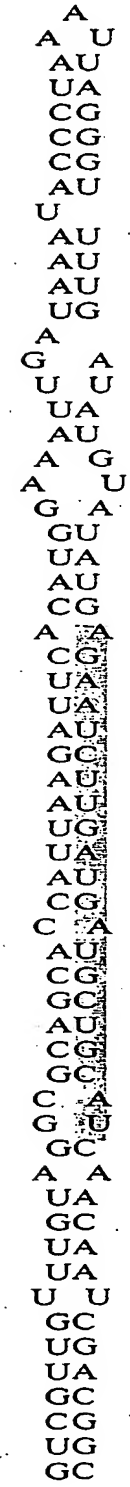
73. The cell of claim 71, wherein the cell is from corn.

74. The isolated polynucleotide of claim 62, wherein the first and the second oligonucleotides are heterologous to the precursor.

75. The isolated polynucleotide of claim 65, wherein the first and the second oligonucleotides are heterologous to the precursor.

76. The isolated polynucleotide of claim 68, wherein the first and the second oligonucleotides are heterologous to the precursor.

FIGURE 1



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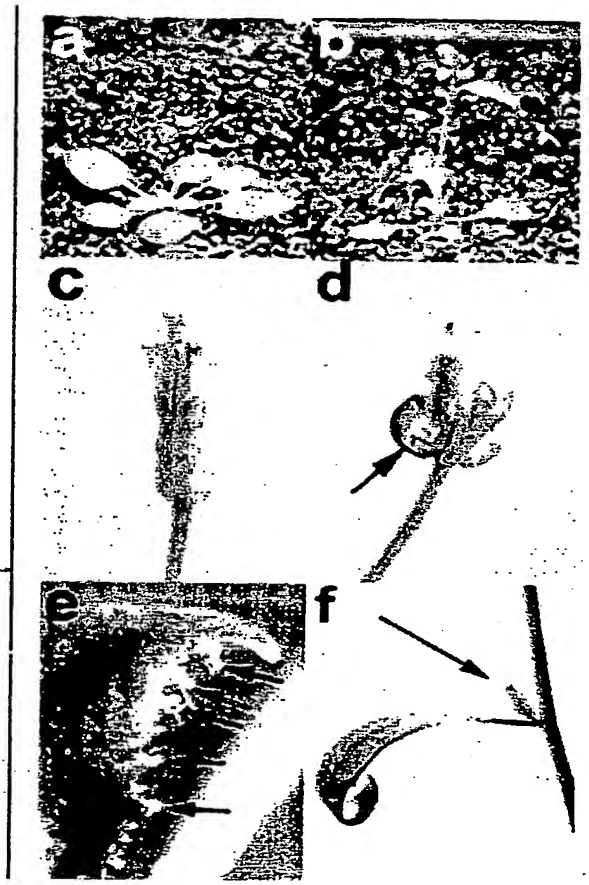

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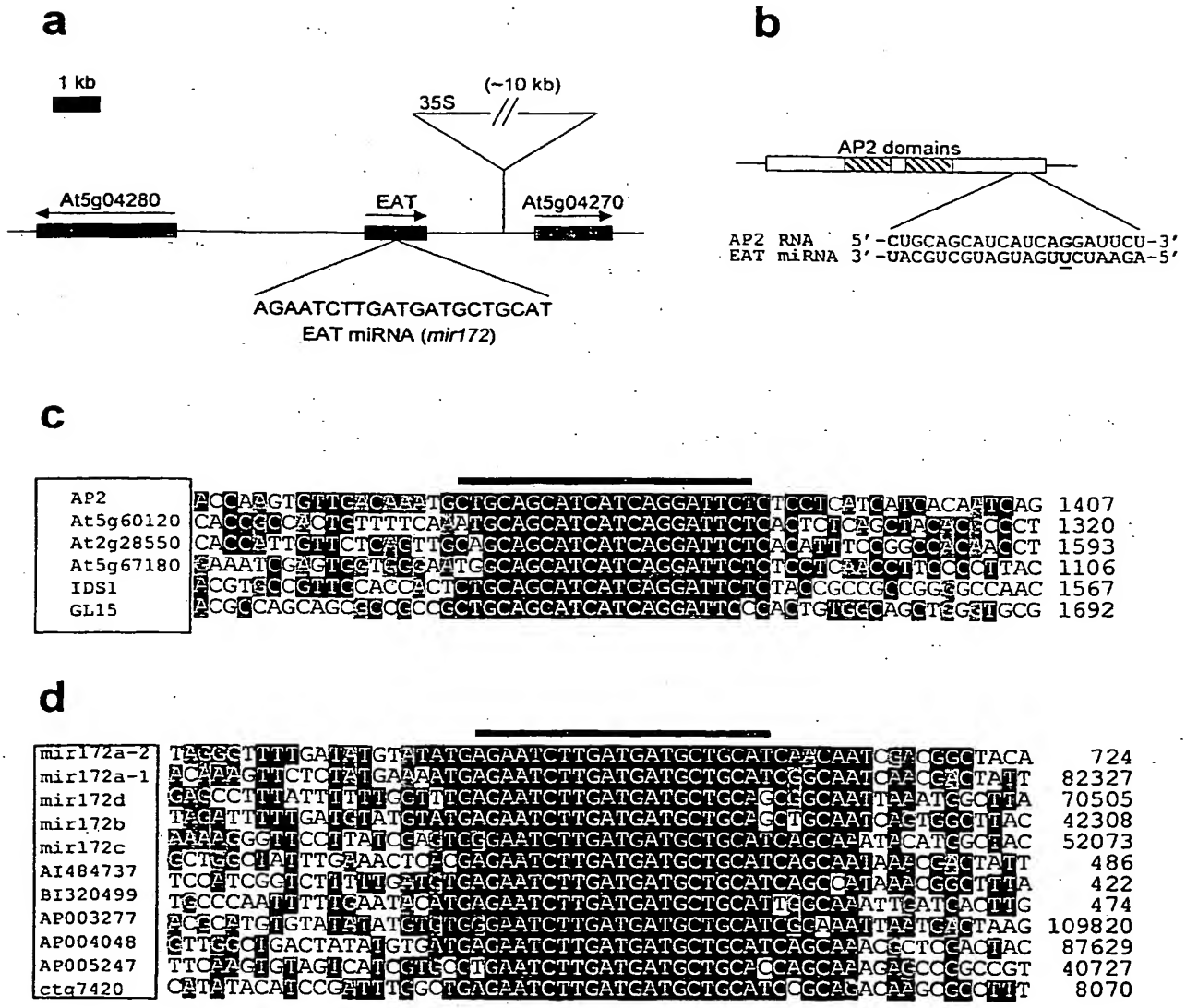

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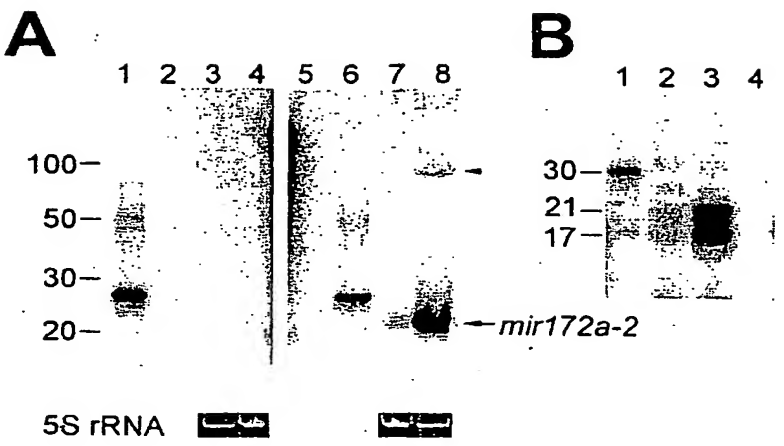

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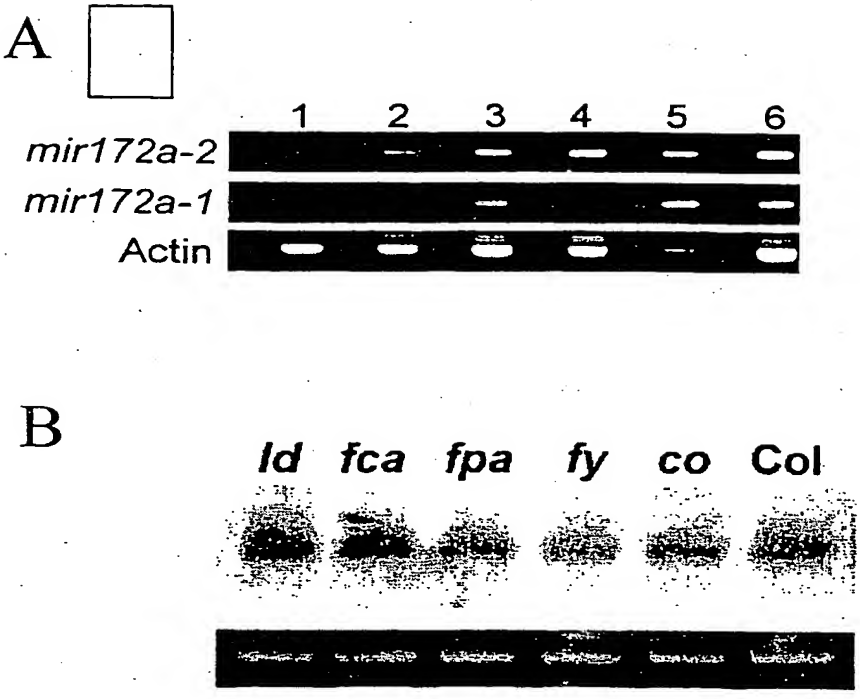

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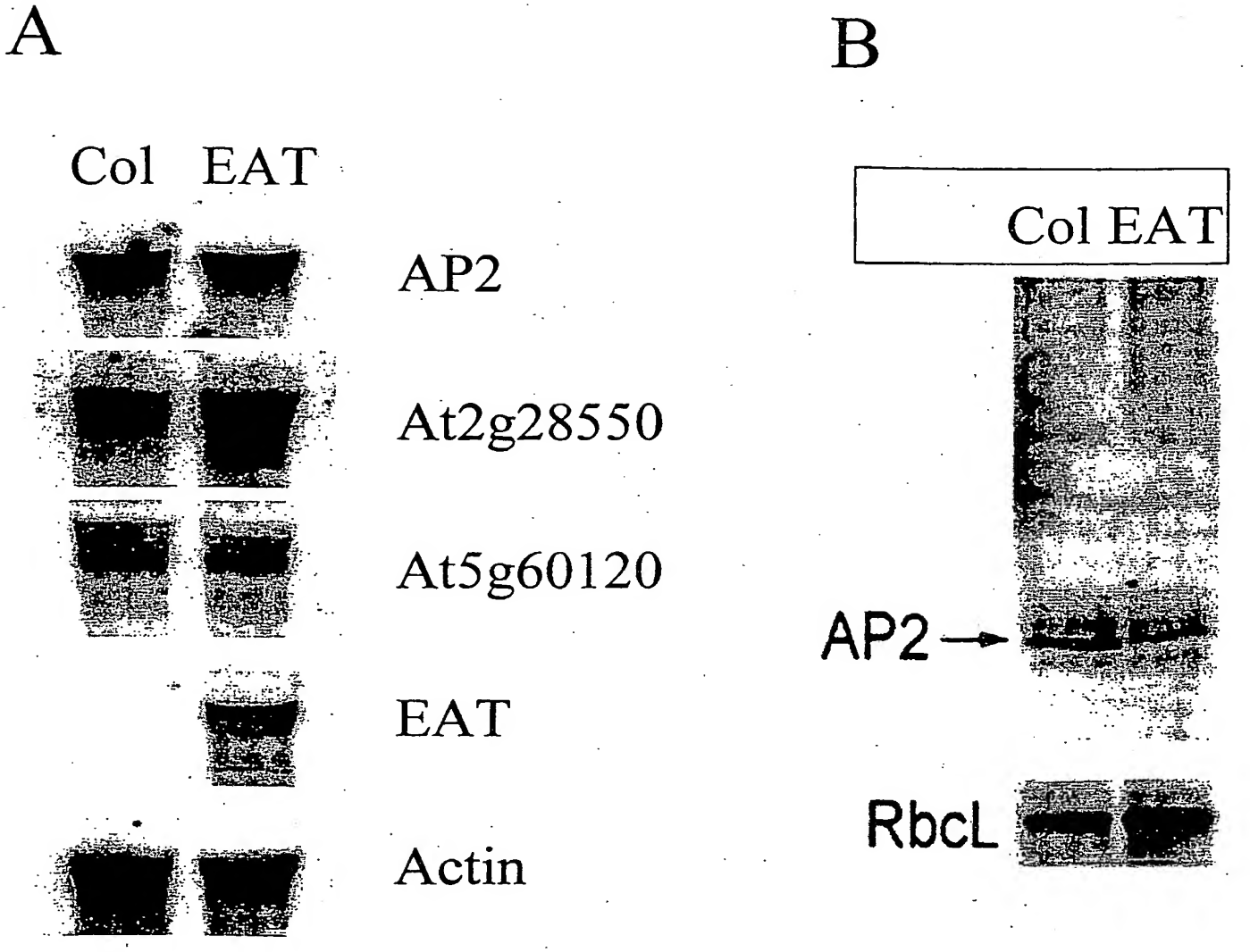

FIGURE 7

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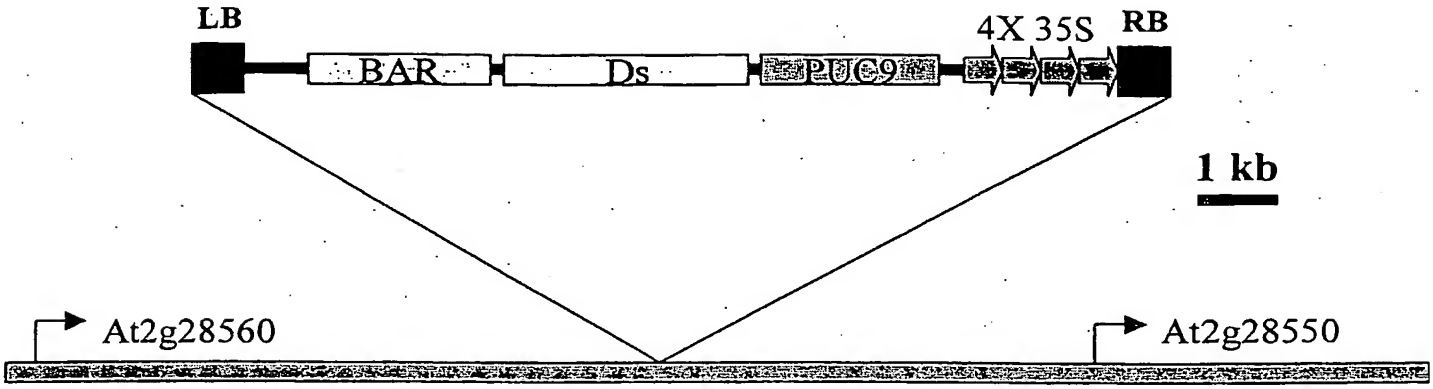
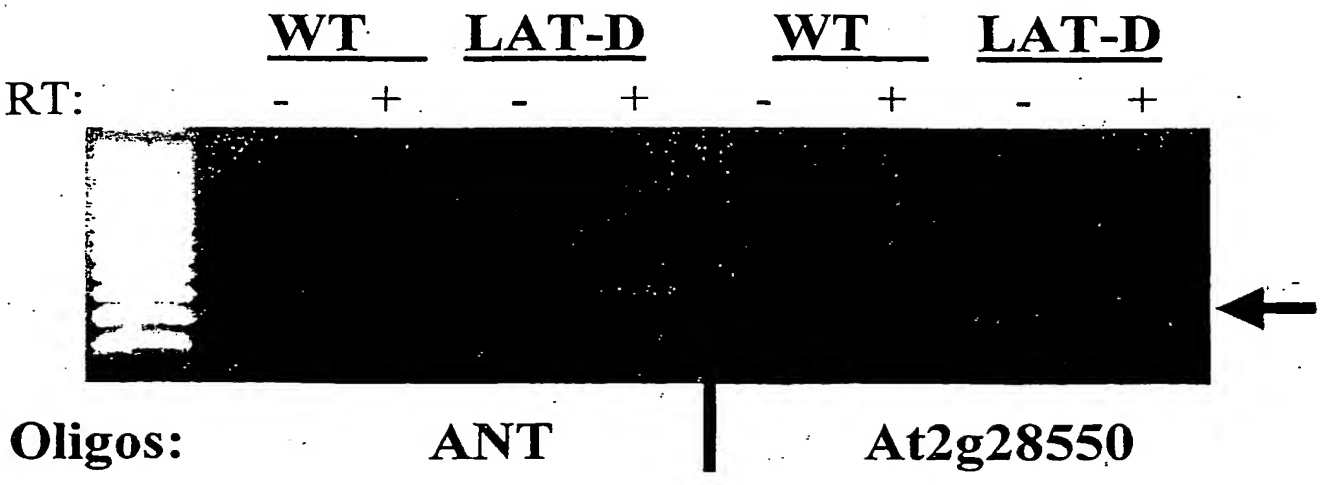


Arabidopsis chromosome 2, BAC T17D12 fragment

B



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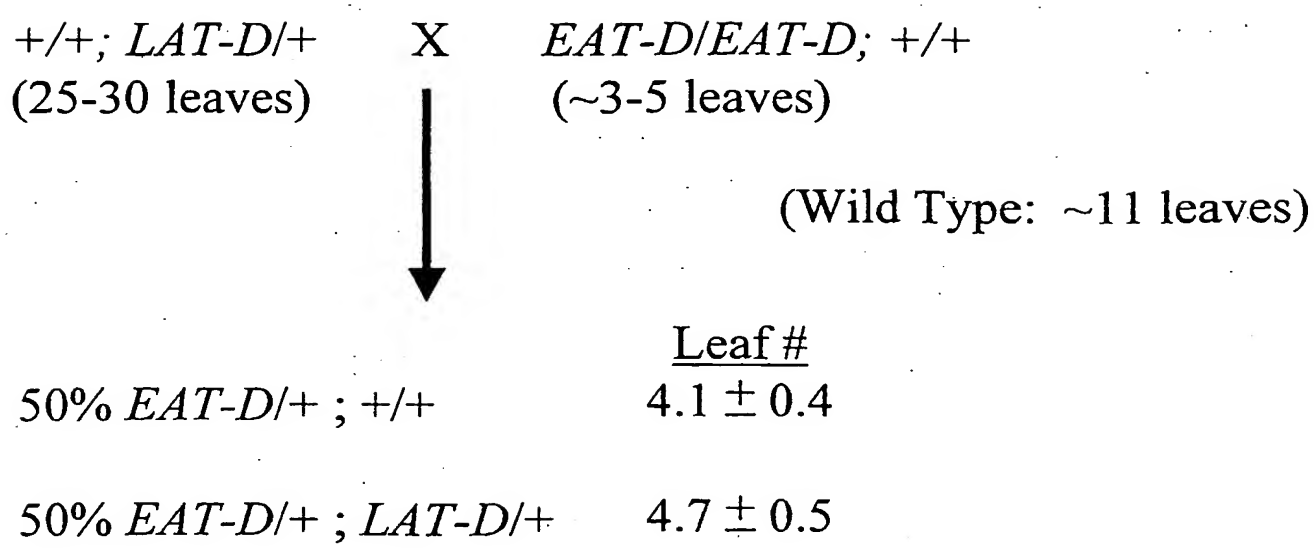

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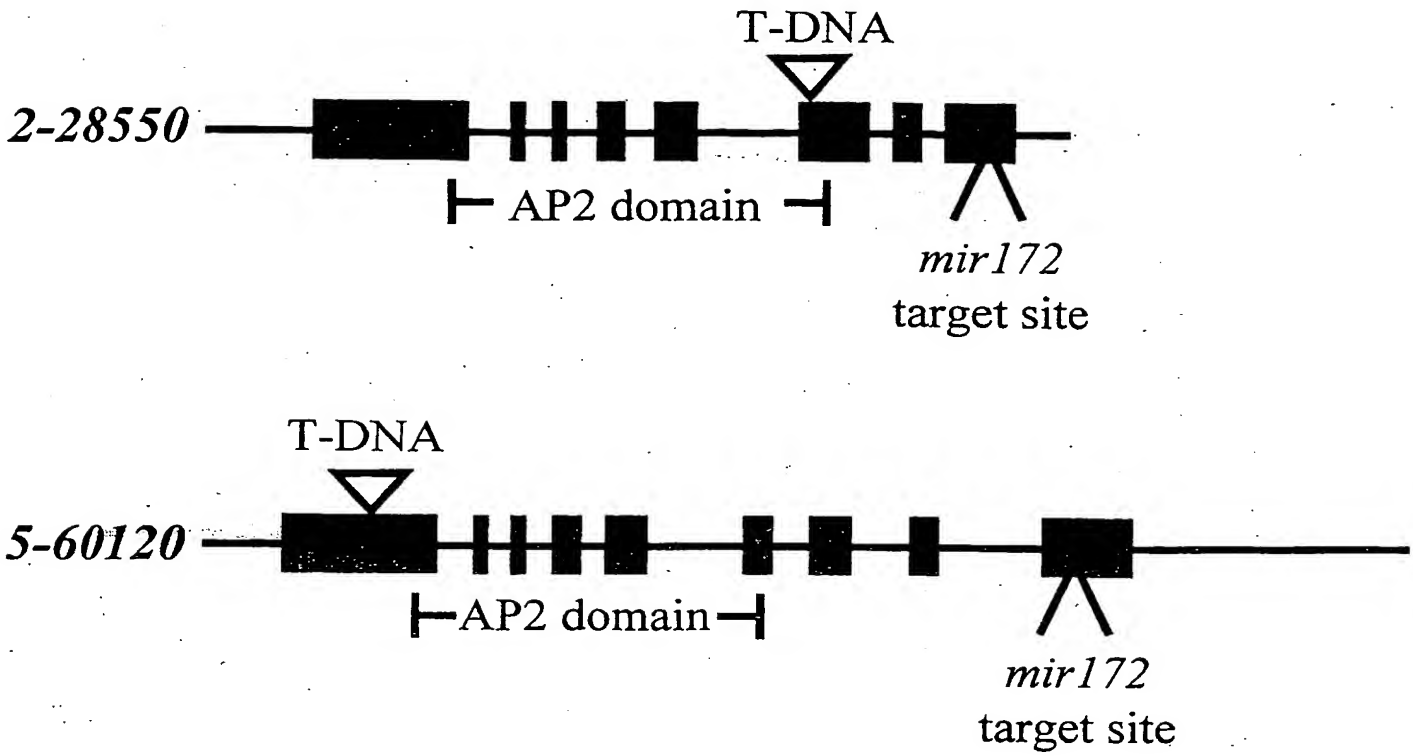
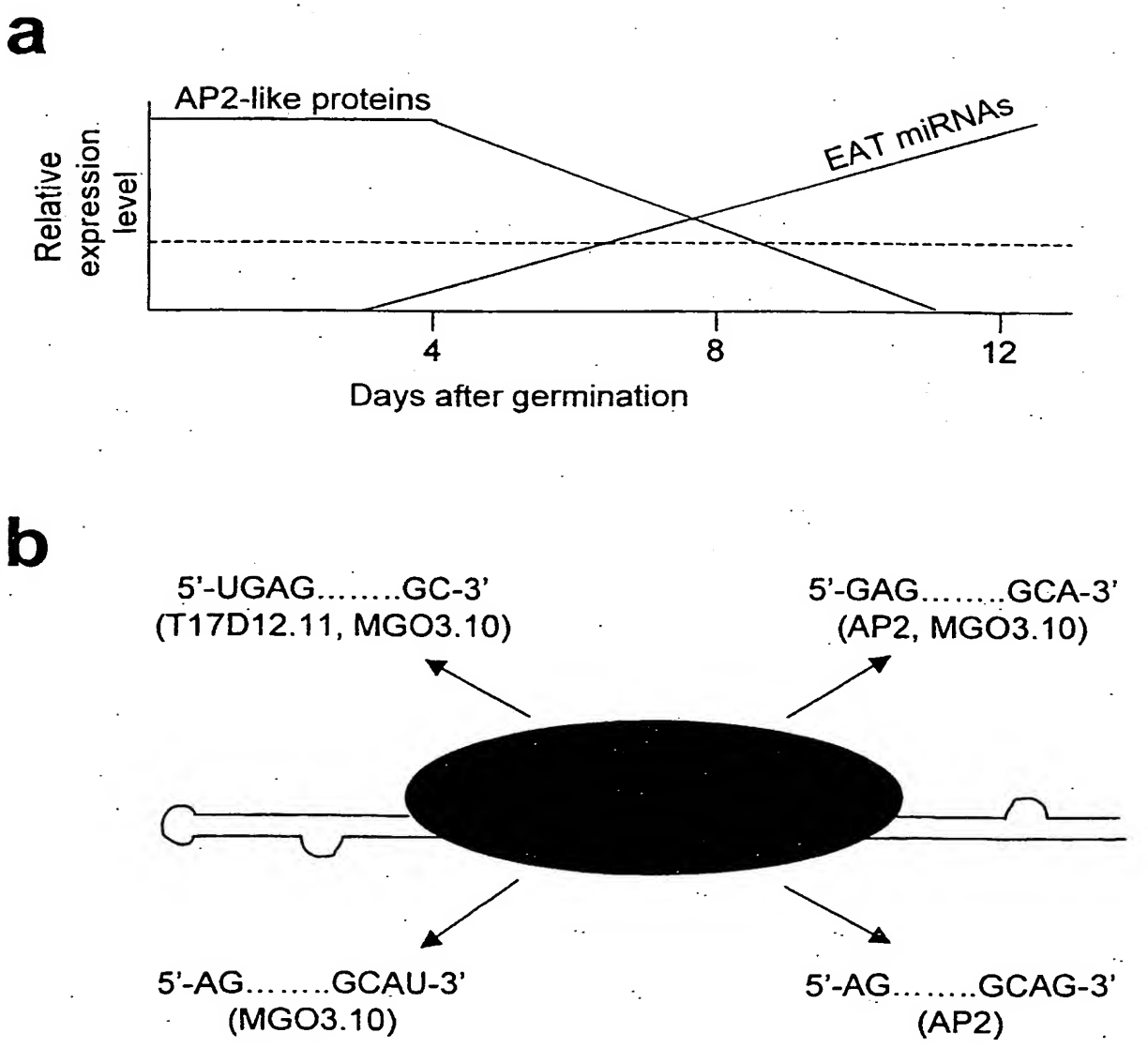


FIGURE 10



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<400> 25
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<210> 26
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<223> Synthetic oligo3/4 for phytoene desaturase target

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ggctacaaat acctaa 76

<210> 27
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<223> Synthetic oligo4/4 for phytoene desaturase target

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tatcaaaacc ctaatt 76

<210> 28
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<220>
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<400> 28
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tggaaattga taaatac 77

<210> 29
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<400> 29
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<210> 30
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<220>
<223> Synthetic oligo3/4 for phytoene desaturase target

<400> 30
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ggctacaaat acctaa 76

<210> 31
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<220>
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<210> 33
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<210> 35
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<210> 36
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<210> 37
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<210> 38
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<210> 40
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<220>
<223> Synthetic oligo1/4 for mi1ps target

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tggaaattga taaatac 77

<210> 41
<211> 77
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<220>
<223> Synthetic oligo2/4 for mi1ps target

<400> 41
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<210> 42
<211> 76
<212> DNA
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<220>
<223> Synthetic oligo3/4 for mi1ps target

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<210> 43
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<212> DNA
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<220>
<223> Synthetic oligo4/4 for mi1ps target

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<220>
<223> EAT 3' PCR primer

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<210> 46
<211> 29
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<220>
<223> EAT 5' PCR primer

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gtcggcggat ccatggaaga aagctcatc 29

<210> 47
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<212> RNA
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<220>
<223> AP2 RNA

<400> 47
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<210> 48
<211> 21
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<213> Arabidopsis thaliana

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<223> EAT miRNA

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accaagtgtt gacaaatgct gcagcatcat caggattctc tcctcatcat cacaatcag 59

<210> 50
<211> 59
<212> DNA
<213> Arabidopsis thaliana

<400> 50
caccgccact gttttcaaat gcagcatcat caggattctc actctcagct acacgccct 59

<210> 51
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<213> Arabidopsis thaliana

<400> 51
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<210> 52
<211> 59
<212> DNA
<213> Arabidopsis thaliana

<400> 52
gaaatcgagt ggtgggaatg gcagcatcat caggattctc tcctcaacct tccccttac 59

<210> 53
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<212> DNA
<213> Zea mays

<400> 53
acgtgccgtt gcaccactct gcagcatcat caggattctc taccgccgcc ggggccaac 59

<210> 54
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<400> 54
acgccagcag cgccgccgct gcagcatcat caggattccc actgtggcag ctgggtgcg 59

<210> 55
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<220>
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gactactcga gcacctctca ctccctttct ctaac 35

<210> 56
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<220>
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<400> 56
gactactcga ggttctcaag ttgagcactt gaaaac 36

<210> 57
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<212> DNA
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<220>
<223> EAT deletion oligonucleotide

<400> 57
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tggaaattga taaatac 77

<210> 58
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<400> 58
cctaaattag ggttttgata tgtatattca acaatcgacg gctacaaata cctaa 55

<210> 59
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<212> DNA
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<220>
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atgagctttc ttccatg 77

<210> 60
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<210> 61
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<210> 62
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<210> 63
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<210> 66
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<223> Actin PCR primer

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atggcagatg gtgaagacat tcag 24

<210> 67
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<400> 67
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<210> 68
<211> 26
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<400> 68
tttccgggca gcagcaacat tggtag 26

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<220>
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gttatagaga gattcattct gtttcacatg 30

<210> 72
<211> 21
<212> DNA
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<220>
<223> miRNA template to EAT

<400> 72
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<210> 73
<211> 175
<212> DNA
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<220>
<223> Synthetic oligonucleotide 1 for EAT with attB sites

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atgatgctgc atcaacaatc gacggcaccc agctttcttg tacaaagtgg tttaa 175

<210> 74
<211> 175
<212> DNA
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<220>
<223> Synthetic oligonucleotide 2 for EAT with attB sites

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<210> 75
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> miRNA template for FAD2

<400> 75
agataagacc aactgtgtca t 21

<210> 76
<211> 175
<212> DNA
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<220>
<223> Synthetic oligonucleotide 1 for FAD2

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<210> 78
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<220>
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<211> 175
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<212> DNA
<213> Zea mays

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(71) Applicant *(for all designated States except US)*: **E. I. DU PONT DE NEMOURS AND COMPANY** [US/US]; 1007 Market Street, Wilmington, Delaware 19898 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **AUKERMAN, Milo J.** [US/US]; 1203 Delpa Drive, Newark, Delaware 19711 (US).

(74) Agent: **DRESS, Virginia M.**; Pioneer Hi-Bred International, Inc., 7100 N.W. 62nd Avenue, Johnston, Iowa 50131-1000 (US).

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[Continued on next page]

(54) Title: GENE SILENCING BY USING MICRO-RNA MOLECULES

(57) **Abstract:** The invention provides methods and compositions useful in target sequence suppression and target sequence validation. The invention provides polynucleotide constructs useful for gene silencing, as well as cells, plants and seeds comprising the polynucleotides. The invention also provides a method for using microRNA to silence a target sequence.

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FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARK WONKEUN ET AL: "CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana" CURRENT BIOLOGY, vol. 12, no. 17, 3 September 2002 (2002-09-03), pages 1484-1495, XP002323690 ISSN: 0960-9822 cited in the application abstract page 1485; figure 1 page 1487; table 1 page 1488; figure 2 ----- -/--	1-76

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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7 April 2005	19/04/2005

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In application No
PCT/US2004/033462

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZENG YAN ET AL: "Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells" MOLECULAR CELL, CELL PRESS, CAMBRIDGE, MA, US, vol. 9, no. 6, June 2002 (2002-06), pages 1327-1333, XP002296481 ISSN: 1097-2765 abstract page 1329; figure 3 pages 1329/1330, bridging paragraph page 1330, left-hand column, paragraph 4 - page 1331, left-hand column, paragraph 1	1-76
X	DATABASE EMBL 16 January 1998 (1998-01-16), "T29B20TF TAMU Arabidopsis thaliana genomic clone T29B20, genomic survey sequence." XP002323692 retrieved from EBI Database accession no. B77795	4,5,9, 11-14,16
A	abstract	1-3,6-8, 10,15, 17-76
P,X	WO 03/093441 A (DUKE UNIVERSITY; CULLEN, BRYAN, R; ZENG, YANG) 13 November 2003 (2003-11-13) page 3, line 8 - line 18 page 30 - page 34; claims 1-30	1-76
P,X	AUKERMAN MILO J ET AL: "Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes." PLANT CELL, vol. 15, no. 11, November 2003 (2003-11), pages 2730-2741, XP002323691 ISSN: 1040-4651 the whole document	1-76

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Application No
PCT/US2004/033462

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		EP 1504126 A2	09-02-2005
		WO 03093441 A2	13-11-2003
		US 2004053411 A1	18-03-2004